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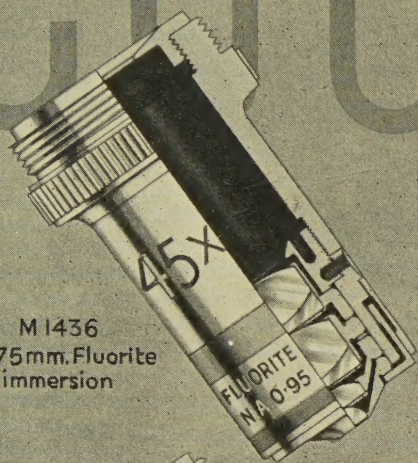
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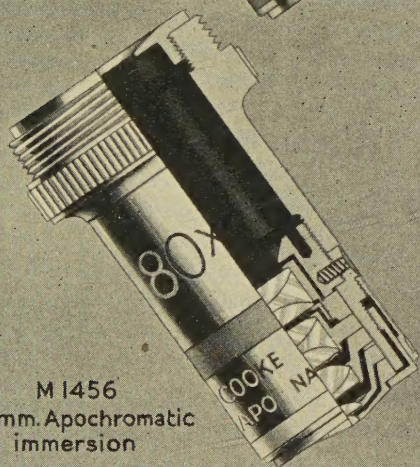
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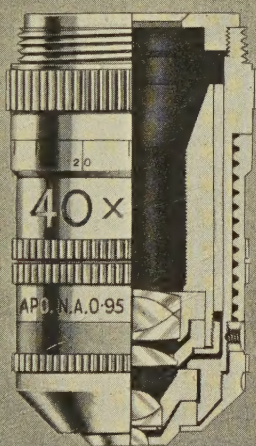
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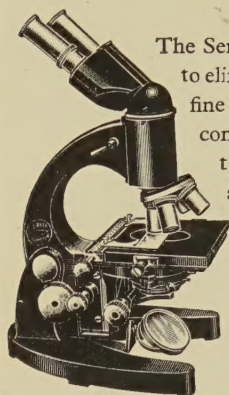


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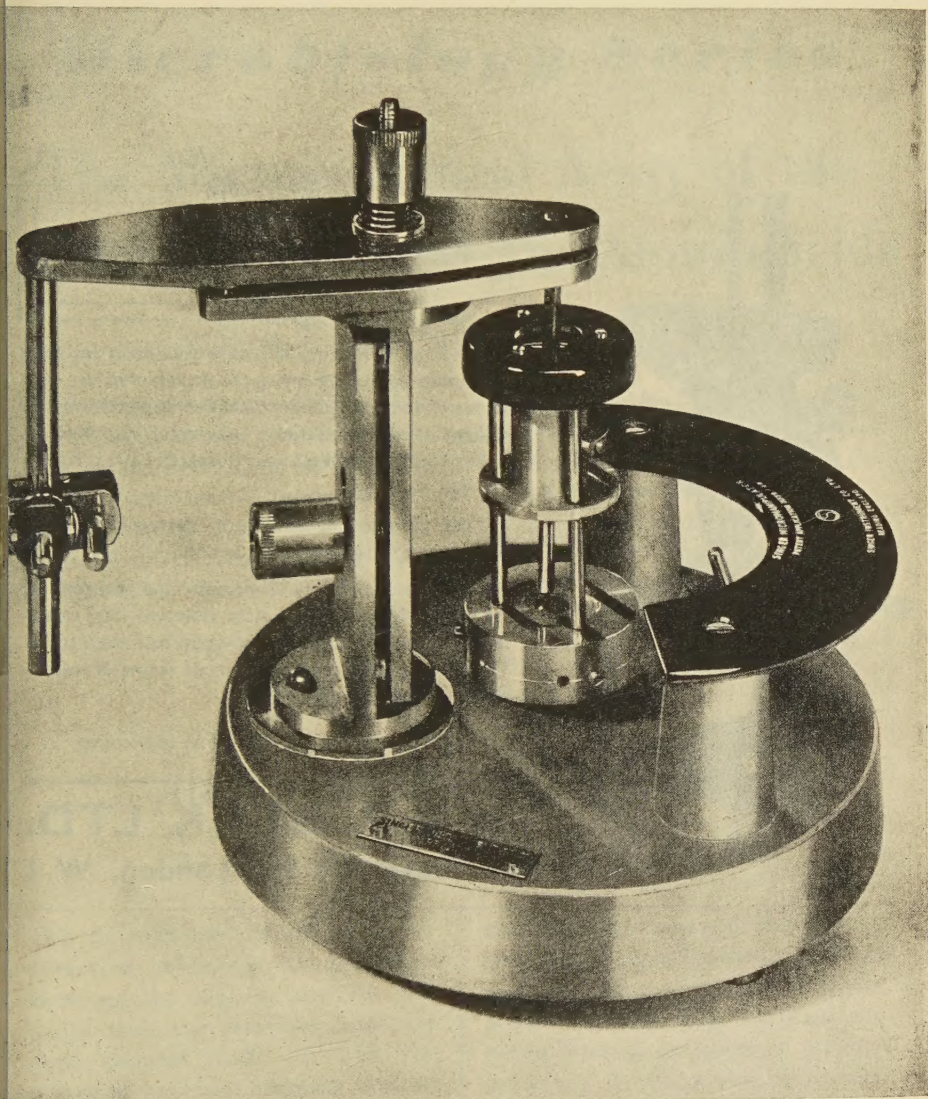
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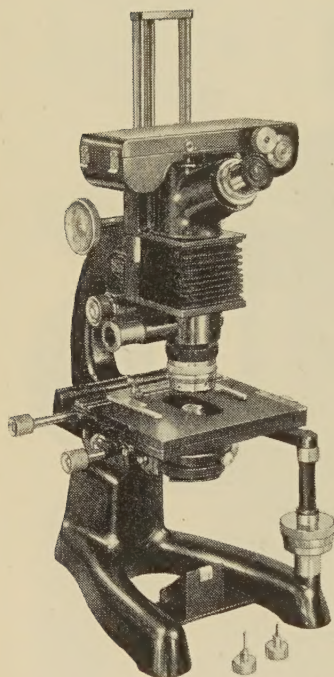
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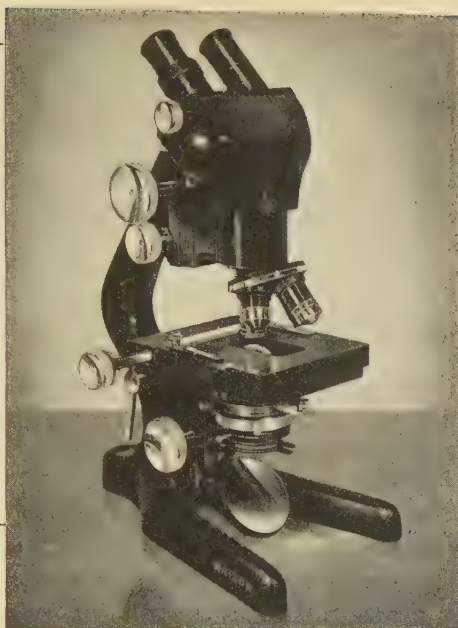
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Remarks on the Effect of the Aperture of the Condenser on Resolution by the Microscope

By JOHN R. BAKER

(From the Department of Zoology and Comparative Anatomy, Oxford)

SUMMARY

Variation of the aperture of the condenser makes somewhat less difference to microscopical resolution than is usually supposed.

IN theoretical discussions of resolution by the microscope, it is conventionally assumed that two points can just be resolved as separate by a particular optical system, when the geometrical image of one of the points lies on the edge of the diffraction-disk representing the other point in the image-plane. The distance apart of the two points when this is so is generally known by the Greek letter ρ . In most books on microscopy ρ is stated to be related to λ (the wave-length of the light used) and to the numerical aperture of the objective thus:

$$\rho = \frac{0.61 \lambda}{n \sin. \alpha}$$

where α is half the angle of the rays accepted by the objective and n the refractive index of the medium in which the angle is measured.

When this equation is used, it is generally understood that the numerical aperture of the condenser is the same as that of the objective. It is often stated that when the two apertures differ, their mean should be used as the denominator of the fraction in the equation. No practical microscopist, however, would accept this. If it were true, one could get *better* resolution with an objective of N.A. 0.1 and a condenser of N.A. 1.2, than with an objective of N.A. 0.7 and a condenser of N.A. 0.5. In fact, of course, the latter arrangement would give quite good resolution and the former very poor.

Hopkins and Barham have recently (1950) provided a theoretical treatment of the influence of condenser-aperture on resolution. They denote by the letter s the N.A. of the condenser divided by the N.A. of the objective. When $s = 1$, the equation given above is correct (on the assumption, always made in discussions of this subject, that perfect spherical correction is achieved in the objective). When s is not 1, however, the figure 0.61 in the equation is no longer applicable. It is changed to a variable which Hopkins and Barham represent by the letter K . They give a graph which shows how K varies with different values of s . The graph is a curved line, crossing the abscissa-value $s = 1$ at the K value 0.61.

Quarterly Journal of Microscopical Science, Vol. 93, part 4, pp. 375-377, Dec, 1952.

The treatment by Hopkins and Barham is quite general. Many microscopists would probably like to know the conclusions to be drawn from their work in terms of practical microscopy, and it is the main purpose of the present paper to provide this information. When an object is too small to be resolved by a particular objective, another of higher aperture is usually substituted until the oil-immersion lens of N.A. 1.3 or 1.4 is reached. These two numerical apertures are therefore of particular importance in matters connected with resolution, and they alone will be considered here. For simplicity, the wave-length of light used may be standardized at 550 m μ (yellowish green). The value of K for different values of s may be obtained from Hopkins's and Barham's graph (their fig. 2). Table 1 in the present paper shows the results of calculations made on this basis.

TABLE 1. *This shows how resolution is affected by the aperture of the condenser.*

N.A. of condenser	ρ (expressed in m μ)	
	with objective of N.A. 1.4	with objective of N.A. 1.3
0.0	327	352
0.1	325	349
0.2	321	344
0.3	315	337
0.4	308	329
0.5	301	320
0.6	293	311
0.7	285	302
0.8	277	294
0.9	270	286
1.0	263	278
1.1	256	270
1.2	250	264
1.3	244	258
1.4	240	253

If a wave-length other than 550 m μ is used, the value of ρ must be multiplied by $\frac{x}{550}$, where x is the wave-length of the light used, expressed in m μ .

The figures in the table show that the influence of the aperture of the condenser on resolution is somewhat less than many microscopists have supposed. For instance, if an objective of N.A. 1.4 is used with a condenser-aperture of 1.4, and the iris diaphragm is then closed to give N.A. 0.7, ρ only changes from 240 to 285 m μ ; or, to express the same facts in another way, the resolution falls from 417 to 351 lines per 100 μ , a drop of only 16 per cent. If one had adopted the old practice of using the mean of N.A. 1.4 and N.A. 0.7 as the denominator of the fraction in the equation $\rho = \frac{0.61 \lambda}{n \sin. \alpha}$, the value of ρ would have increased to 320 m μ and the resolution would have fallen to 313 lines per 100 μ , 25 per cent. (instead of 16) below the full-aperture figure.

It must be remarked that ordinary microscopical images are affected by a factor separate from those concerned in the theoretical basis of the table. If any object is of markedly different refractive index from the surrounding mounting-medium, it will tend (as is well known) to appear with a Becke line round it, and this will necessarily interfere with the perfection of the image. The effect will be increased if a narrow cone of light is used for illumination, whether that cone is central (that is, central light of low numerical aperture) or not (oblique light of high numerical aperture). Whenever one opens the iris diaphragm, this undesirable Becke effect will be reduced.

There are theoretical reasons, however, for supposing that it is usually undesirable to use an illuminating cone of quite so high a numerical aperture as the objective, even if the extreme marginal zone of the latter be assumed to be perfectly corrected for spherical aberration (see Oettlé, 1950). This accords with the experience of most practical microscopists. Oettlé's explanation is that when direct light enters the marginal zone of an objective, only half of the diffracted rays are concerned in image-formation, the remainder being too oblique to enter the lens-system.

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- HOPKINS, H. H., and BARHAM, P. M., 1950. *Proc. Phys. Soc. B*, **63**, 737.
OETTLÉ, A. G., 1950. *Quart. J. micr. Sci.*, **91**, 348.

A simple Technique for direct Observation of the Effect of Radiation on living Cells *in Vitro*

By L. G. LAJTHA, M.D., D.PHIL., and R. OLIVER, M.Sc., A.INST.P.

(From the Department of Radiotherapy, Churchill Hospital, Oxford)

SUMMARY

A chamber with glass coverslip at top and bottom is filled with a beta-emitting radioactive isotope (e.g. P^{32}). A drop of cell-suspension placed on top of the chamber may then be irradiated at dose-rates up to 20,000 rep/hour, while the optical conditions remain unimpaired for microscopical examination. Extra protection for the observer is unnecessary.

A TECHNIQUE has been devised in our laboratory for irradiating living cells while they are being observed under the phase-contrast microscope. The technique can be used immediately with any laboratory microscope, and any dose-rate up to at least 20,000 rep/hour can be delivered to the cells without any danger to the observer even during several hours of continuous examination.

A special chamber, already described and figured by one of us (Lajtha, 1950), is used in this technique. It consists of a ring of solid metal (chromium-plated), with a cover-glass sealed on to each side of it with paraffin wax (m.p. 56°C.). The sealing is done thus. The ring is dipped in molten wax, allowed to cool, and then placed on a coverslip. It is then warmed with a flame and the second coverslip is dropped on top to seal the chamber. The radioactive solution is injected into the chamber through one of the two small holes that pass through the thickness of the metal ring (see the paper quoted above); these holes, which admit a No. 16 needle, are afterwards sealed with paraffin wax. (The chamber can, if necessary, be mounted on a microscope slide to fit the moving stage of the microscope.)

One drop of cell-suspension is placed on top of the upper coverslip, covered with a third coverslip, and sealed off. Thus the optical conditions remain unimpaired, and the cells are separated from the radioactive solution only by a coverslip $\frac{1}{8}$ mm. thick. When a radioactive isotope that emits beta rays only is used (e.g. P^{32} , Sr^{90}), these rays are able to penetrate the thin coverslip and irradiate the cells; but the metal wall of the chamber and the microscope objective and condenser prevent the electrons from reaching the observer, without the necessity for the bulky lead protection required with gamma ray sources.

From a knowledge of the radiation energy it is possible to calculate the dose-rate at the cells to within about 10–15 per cent. This is usually sufficient [Quarterly Journal of Microscopical Science, Vol. 93, part 4, pp. 379–380, Dec. 1952.]

for this type of work, but if greater accuracy is required it is possible to measure the dose-rate at the coverslip surface with a thin-walled ionization chamber. At present we have used radioactive phosphorus (P^{32}), which has a convenient half-life of 14 days and is readily available from Harwell in any desired activity up to 30 mc./ml. This will provide a dose-rate of about 18,000 rep/hour at the cells during the first day, and the source may be kept for use at lower dose-rates as the phosphorus decays.

A thick-walled Petri dish provides adequate protection for the storage of these chambers, which may thus be easily and safely transported.

This work is part of an investigation carried out under a grant from the British Empire Cancer Campaign.

REFERENCE

- LAJTHA, L. G., 1950. *Quart. J. micr. Sci.*, **91**, 107.

Observations concerning the Specificity of the Acid Haematein Test for Phospholipines

By W. G. BRUCE CASSELMAN

(Medical Research Fellow, National Research Council, Canada)

With the Technical Assistance of

O. T. GEORGE

(From the Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada)

SUMMARY

Studies with synthetic enantiomeric α -phosphatidic acids, α -phospholipines, and related compounds, and with highly purified, natural lipine fractions confirm that Baker's 'acid haematein test' (controlled by the pyridine extraction test) is a method for the histochemical recognition not of lipine in general, but of phospholipine'. A true positive reaction is also given by certain phosphatidic acids.

INTRODUCTION

THE acid haematein test, introduced by Baker in 1946, is considered to be the only satisfactory method for the histochemical recognition of phospholipines (Cain, 1950). Initially, Baker believed that his procedure demonstrated all classes of lipines but, later, he established that it was specific for the phospholipines (Baker, 1946, 1947). This was confirmed by Cain (1947) while working in Baker's laboratory. In assessing the validity of the acid haematein test, both of these investigators had to depend upon the reactions given by lipine fractions isolated from natural sources rather than upon those given by compounds of completely known chemical structure. Apparently, they did not test any phosphatidic acids.

In this department, Baer and his associates have succeeded in totally synthesizing various enantiomeric α -phosphatidic acids and α -phospholipines (Baer, 1951; Baer and Maurukas, 1952; Baer, Maurukas, and Russell, 1952). Thus has been afforded an excellent opportunity for the study of the specificity of the acid haematein test with substances of established constitutional and configurational identity. This paper reports the results of such a study employing a variety of synthetic compounds, generously provided by Professor Erich Baer, and certain highly purified, natural lipine fractions supplied by Dr. L. B. Macpherson.

METHODS

In most instances, the tests were made on strips of thin cigarette paper impregnated with various pure substances or mixtures, as recommended by [Quarterly Journal of Microscopical Science, Vol. 93, part 4, pp. 381-383, Dec. 1952.]

Baker (1946). His staining schedule was followed closely because both he and Cain (1947, 1950) have stressed the importance of precise timing. Gelatine-embedding and subsequent sectioning were usually omitted. Pyridine extraction tests (Baker, 1946) were run concurrently. A further control was provided by final coloration of all papers with oil red O to detect any sudanophilic material not stained by the acid haematein. To make the observations as objective as possible, the treated papers were examined at random and their identities remained unknown to the observer until all of the results had been recorded. The colour reactions were interpreted in strict conformity with the recommendations of Baker (1946, 1947) and Cain (1947, 1950).

OBSERVATIONS AND DISCUSSION

The reactions given by the pure, synthetic α -phosphatidic acids, α -phospholipines and related compounds and by the highly purified, natural lipines are presented in Table 1. As shown there, some of the synthetic substances are soluble in the reagents used for the acid haematein test. However, true positive reactions (AH+, PE—) are given by the free, synthetic phosphatidic acids and by the synthetic and natural lipines. Completely negative reactions (AH—, PE—) are given by the synthetic triglycerides and by the natural cerebrosides.

TABLE 1

<i>Substance</i>	<i>AH</i>	<i>PE</i>
<i>Synthetic compounds:</i>		
Glycerophosphoric acid, lithium salt	S	S
Phosphorylcholine chloride, barium salt	S	S
L- α -glycerylphosphorylethanolamine monohydrate	S	S
Distearoyl-L- α -glycerophosphoric acid, ethanolamine salt	S	S
Dimyristoyl-L- α -glycerophosphoric acid, choline salt	S	S
Distearoyl-L- α -glycerophosphoric acid	—	—
Tetramyristoyl bis(L- α -glyceryl) phosphoric acid	—	—
L- α -(dipalmitoyl) lecithin	—	—
L- α -(dimyristoyl) lecithin	—	—
L- α -(distearoyl) cephalin	+	—
Trimyristin	—	—
Tripalmitin	—	—
Tristearin	—	—
<i>Highly purified, natural lipine fractions:</i>		
Cephalins (brain)	+	—
Lecithins (brain)	+	—
Sphingomyelins (brain)	+	—
Lipositols (liver, soya bean)	+	—
Cerebrosides (brain)	—	—

Symbols and abbreviations: AH = acid haematein test; PE = pyridine extraction test; S = soluble in reagents; + = positive reaction; — = negative reaction.

An additional 123 other substances, many of them previously studied by Baker (1946) and Cain (1947), were tested but none gave true positive re-

actions. These included a wide variety of other lipoid and non-lipoid materials representing aliphatic and other hydrocarbons; alcohols and phenols; ethers; fatty and other organic acids, their esters and soaps; mono- and triglycerides; sterols and their esters, tocopherols; animal and vegetable fats and oils; essential oils; mono-, di-, and polysaccharides; various simple, conjugated and derived proteins; amino acids; purine and pyrimidine compounds; and a few miscellaneous substances.

These observations independently confirm Baker's (1947) claim that 'the acid haematein test (controlled by the pyridine extraction test) is a method for the histochemical recognition not of lipine in general, but of phospholipine'. However, they suggest that the entire phospholipine molecule may not be essential because some phosphatidic acids also give true positive reactions. This is being studied further.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the inspiration and guidance of Professor C. H. Best.

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Observations upon the Recovery of Oxyntic Cells after Prolonged Dosage with Pilocarpine or Histamine

By GORDON MENZIES

(From the Department of Anatomy, St. Mary's Hospital Medical School, Paddington, London, W. 2)

With one plate (fig. 1)

SUMMARY

1. New observations are presented concerning the structure and cytochemistry of the granules in the oxyntic cells of the rat's stomach, in continuation of work already reported (Menzies, 1949, 1952 *a* and *b*).
2. The phospholipine component of the granules is slowly regained after being depleted by multiple doses of pilocarpine or histamine.
3. The phospholipine is first regained by the granules in those oxyntic cells situated at the basal ends of the gastric tubules, and later by the remaining cells in the neck of the tubules.
4. An acid phosphatase appears in the oxyntic cells whose granules are about to regain their lipine component, and disappears some time after they have done so.
5. The order in which both the acid phosphatase and the lipine appears (first in the basal cells and later in cells in the neck region) is the same as that in which an acid phosphatase appears when and as the lipine is being shed under experimental conditions (Menzies, 1952 *b*).

INTRODUCTION

IN a recent series of papers it has been shown that the oxyntic granules in the rat's stomach contain a lipid and a non-lipid component (Menzies, 1949), and that the lipid component consists partly of a phospholipine and partly of non-lipine lipid (Menzies, 1952 *a*). It was also shown that during the course of repeated injections of pilocarpine or histamine the phospholipine is slowly shed from the granules, while acid phosphatase activity appears in the oxyntic cell (Menzies, 1952 *b*).

The purpose of this paper is to investigate further the effects of multiple injections of pilocarpine and histamine on the oxyntic cells and to describe the processes of recovery from these effects after cessation of injections.

MATERIALS AND METHODS

In this investigation twenty male Norwegian rats each of approximately 200 gm. body weight were used. They were all fed with oatmeal, dried milk, and cabbage, put in separate cages and starved for 24 hours, during which time drinking water was freely available. Four were then killed as controls. Only four controls were considered necessary because (as proved to be the case) the results of the controls were similar to those of the experimental rats. (*Quarterly Journal of Microscopical Science*, Vol. 93, part 4, pp. 385-390, Dec. 1952.)

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case) they differed in no appreciable way from similar animals already described (Menzies, 1949).

Twelve of the remaining rats were given twelve intraperitoneal injections of pilocarpine nitrate (25 mg./kg. in 1 c.c. distilled water) at half-hourly intervals. They were killed in batches of three, half an hour, 5 hours, 8 hours and 20 hours after their twelfth injection. Four rats were given twelve intraperitoneal injections of histamine hydrochloride (5 mg./kg. in 1 c.c. distilled water) at 45-minute intervals. They were killed 45 minutes, 5 hours, 8 hours and 24 hours after their final injection.

Histochemical and cytological techniques

As these were similar to those used in the author's previous papers mentioned above, they will not be detailed here. In all cases small pieces of the mucous membrane from the fundus of the stomach were subjected to the following tests:

For lipoids in general. Sudan black (Baker, 1944).

For phospholipine. The acid haematein test (Baker, 1946).

Further, after the removal of lipoids by hot pyridine, the remaining non-lipoid moiety of the granules was stained by Heidenhain's iron haematoxylin.

For enzymes. (1) Lipase (Gomori, 1946); (2) alkaline phosphatase (Gomori, 1939); (3) acid phosphatase (Gomori, 1941).

RESULTS

Control rats

All the granules in the vast majority of the oxyntic cells colour black or bluish black with acid haematein (fig. 1, A). In a small proportion of cells always situated at the basal ends of a few of the gastric tubules, some or all of the granules fail to colour with acid haematein, but they are still visible by virtue of their refractility and can be demonstrated with Sudan black and Heidenhain's iron haematoxylin. I would suggest that the cells containing granules uncoloured by acid haematein are either still actively secreting, or have secreted more recently than the remaining cells whose granules colour. All the granules in all the oxyntic cells colour with Sudan black. When the lipoids have been removed by hot pyridine all granules in all the cells stain with Heidenhain's iron haematoxylin. Apart from a trace of alkaline phosphatase in the oxyntic nuclei, neither lipase nor alkaline phosphatase is present in the control animals. Acid phosphatase is absent, never having been seen in the numerous sections tested.

Rats after twelve injections of pilocarpine or histamine

After pilocarpine none of the granules in any of the oxyntic cells colour with acid haematein (fig. 1, B) although they can still be seen by virtue of their refractility. That this is not due to a failure of the haematein is shown by the fact that other structures in the mucosa that normally colour with acid

haematein still do so to the same intensity (fig. 1, B). After histamine a few cells in the neck region of the tubules show a few granules faintly coloured by acid haematein. In marked contrast to this, after both pilocarpine and histamine all the granules in all the oxyntic cells colour with Sudan black (fig. 1, C). After pilocarpine Sudan black shows a marked clumping of granules, and individual granules cannot always be resolved (the condition resembling that which has been seen when using acid haematein on the oxyntic cells of 20-day-old rat embryos). The granules appear larger than in the controls, are distorted and irregular in shape, and lack homogeneity in intensity of colouring. After histamine and colouring with Sudan black the granules are smaller than in the controls, and they are more separate than after pilocarpine, though not so separate as in the controls. Heidenhain's iron haematoxylin shows that the non-lipoid moiety of the granules is indistinguishable from the controls. The tests for the enzymes were negative, as in the controls.

Rats killed from 5 to 24 hours after injection

The results after pilocarpine are so similar to those after histamine that they will be described together. The general picture is of a progressive reappearance of phospholipine in the granules and the appearance of acid phosphatase activity, both processes beginning at the base of the glands and advancing towards the lumen. There is a general correspondence between the position of the cells showing enzyme activity and that of the cells whose granules show returning coloration with acid haematein.

All the animals killed after 5 hours show a band of oxyntic cells situated in the basal half of the tubules, whose granules colour either faintly or darkly with acid haematein, indicating a reappearance of phospholipine. In sections where the basal granules are only faintly coloured by acid haematein, the granules of the more superficial cells are uncoloured. In sections where the granules are deeply coloured in the basal cells, a few scattered cells in the necks of the tubules are coloured, usually faintly but sometimes deeply (fig. 1, D). In the above animals an acid phosphatase is present in a similar band of oxyntic cells in the basal half of the tubules (fig. 1, E). Although the acid phosphatase is largely confined to the nuclei, granules, and cytoplasm of the oxyntic cells, other nearby cells sometimes show the enzyme as well, such as peptic cells, fibroblasts, and vascular endothelium. Occasionally in phosphatase preparations the intracellular canals of the oxyntic cells are visible as clear areas against a background of darkly stained cytoplasm.

In rats killed 8 hours after the last injection acid haematein shows (fig. 1, F) that the granules in the most basally situated oxyntic cells colour almost as deeply as in the controls, while the granules in the other oxyntic cells all colour, but less deeply. This results either in the appearance of two bands, as seen under low power, viz. a basally situated dark band and a more superficially situated lighter band, or in a gradual merging of a darker basal area into a lighter superficial area. In all the animals killed after 8 hours, except one, acid phosphatase is present in a band of oxyntic cells situated in the neck

region of the tubule (fig. 1, G). In one animal acid phosphatase is present in all structures (not only oxyntic cells), in the neck region of the tubules, and in addition some of the oxyntic cells more basally situated show the presence of the enzyme. This animal shows a stage intermediate between those of figures 1, E and 1, G, and indicates that the enzyme appears in the luminal band before it has completely disappeared from the basal band. Furthermore, in this animal the phosphatase activity towards the lumen of the stomach is present in all structures adjacent to the oxyntic cells, as well as in those cells (The oxyntic cells here, in the rat, form a wide band from end to end of the mucosa.) It may well be that in this case the addition of the lipine was proceeding very rapidly, for I have often noticed similar appearances in animals where the granules were shedding the lipine within a matter of seconds, viz. following mecholyl, insulin, and after a very small dose of pilocarpine in animals whose granule morphology has been rendered unstable by prolonged starvation (unpublished work).

In all the above animals killed after 5 or 8 hours there is no evidence of any granules in the oxyntic cells which do not colour with Sudan black. The granules that colour with acid haematein are smaller than those in the controls. There is no evidence of the elongation of granules that occurs after a single injection of histamine, and no uncoloured central areas are seen (as characteristically after a single injection of pilocarpine). Also in these groups Heidenhain's iron haematoxylin stains the non-lipoidal component of the granule in a manner indistinguishable from that of the controls.

In the four rats killed 24 hours after the final injection, acid haematein and the other tests mentioned give results similar to those in the controls; in particular acid phosphatase was never demonstrated in any of the numerous sections tested. This result indicates that recovery from the effects of multiple doses of pilocarpine and histamine occurs in less than 24 hours in the rat.

FIG. 1. The luminal side of the gastric mucosa is towards the top of the photomicrograph in all cases. All scales = 50 μ .

A, control rat, showing mucosa coloured by acid haematein (gelatin-frozen section). The oxyntic cells are packed with darkly coloured granules. Mucin towards the lumen, and red blood-cells at the bottom of the photograph also colour.

B, acid haematein preparation of rat killed half an hour after twelve injections of pilocarpine. There is no evidence of colouring in the oxyntic cells, though red blood-cells and tissue eosinophils colour as in the controls.

C, gastric mucosa of the same rat as B, coloured with Sudan black.

D, rat killed 5 hours after injections (acid haematein). The oxyntic granules in many of the basal cells colour, as do some in a few cells more superficially placed. Much mucin is seen in the gastric crypts towards the lumen.

E, a similar animal to D, showing acid phosphatase in the basal cells. The counterstain with safranin, which shows up the nuclei in the peripheral mucosa.

F, acid haematein preparation of rat killed 8 hours after injections, showing the return of phospholipine to the oxyntic granules of the superficially placed cells. Note that mucin also colours back.

G, the same animal as F, showing acid phosphatase in the superficial oxyntic cells. Counterstained with safranin.

DISCUSSION

The above results indicate that the changes induced within the oxyntic cells of the rat's stomach by repeated doses of pilocarpine or histamine disappear within 24 hours of the cessation of the injections.

Repeated injections of either drug cause a gradual shedding of the phospholipine moiety of the oxyntic granules, starting in the basal cells and gradually spreading towards the lumen (Menzies, 1952 *b*), until eventually, as shown by the present work, all the granules have lost their phospholipine component. The granules, however, are still visible by virtue of their refractility, and Sudan black shows that they still contain a non-lipine lipid. After removal of this lipid by immersion in hot pyridine, staining with iron haematoxylin demonstrates that the non-lipoid component (presumably containing protein) is still present. During the shedding of phospholipine (Menzies, 1952 *b*) acid phosphatase activity becomes demonstrable in a progressive manner, starting in the basal cells and advancing towards the lumen at the same rate as the release of phospholipine. The present results show that phosphatase activity disappears after twelve injections of the drugs, when all the phospholipine has been shed.

After a period of rest the phospholipine component of the granules is gradually regained, first by those granules situated in the basal cells and later by those in cells more superficially placed, until a stage is reached when they cannot be distinguished from the normal controls. Associated with the return of the phospholipine to the granules, acid phosphatase activity reappears, both processes beginning in the basal cells and advancing towards the superficial cells. There is evidence that the enzyme reappears a little before the return of the phospholipine, so that it is present before as well as during the addition of this component to the granules. From the present work and that previously reported, it is clear that both the disappearance and reappearance of phospholipine takes place in an orderly, progressive manner from the base of the tubules towards the lumen, and that during both processes acid phosphatase activity is demonstrable along a similar gradient. The orderly disappearance and return of phospholipine in the mucosa suggests the existence of a differential sensitivity of the oxyntic cells to the operation of secretagogues. Regarding the association of phosphatase activity with the release of phospholipine, the present writer has already suggested (1952 *b*) that the enzyme may be responsible for hydrolysing the phospholipine, and further support for this is shown by the present work, for enzymes that will hydrolyse a substance will under other conditions synthesize it, and possibly that is its role in the present experiments. It must be borne in mind, however, that the acid phosphatase demonstrated during the release of phospholipine may not necessarily be the same enzyme as that demonstrated during its reformation. The phosphatase activity shown in the present work may simply reflect the increased rate of phosphatase turnover such as is known to exist in many cells during their recovery from injurious stimuli. The type of phosphatase is a matter of speculation, that

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which occurs during the release of phospholipine is likely to be a di-esterase such, for example, as lecithinase, for a mono-esterase would not hydrolyse phospholipine.

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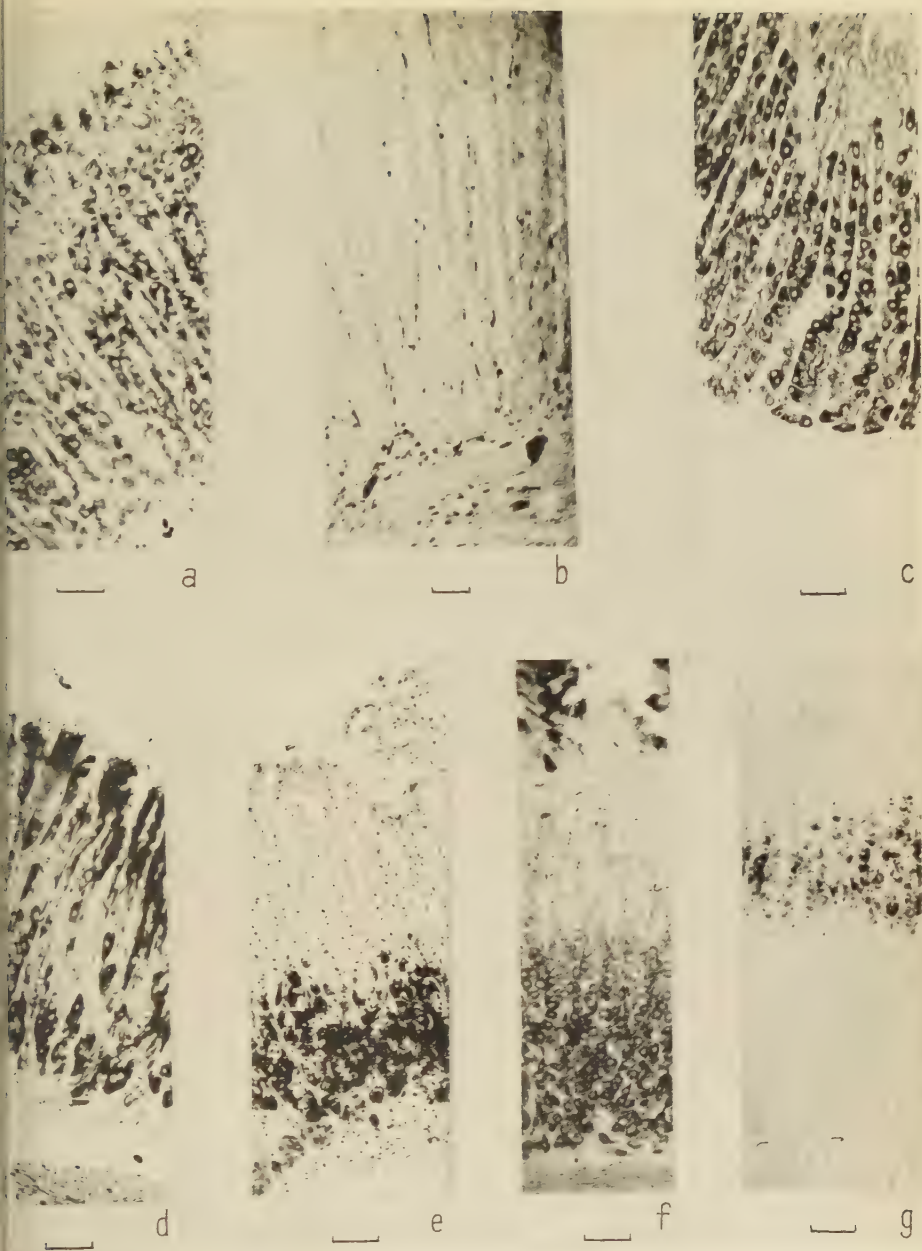


FIG. 1
G. MENZIES

Histochemical Distribution of 5-Nucleotidase in Snake Tissues: Presence of 5-Nucleotidase and Absence of Alkaline Phosphomonoesterase in Venom Glands of the Rattlesnake

By D. E. BRAGDON AND J. F. A. McMANUS

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Department of Medicine, Charlottesville, Virginia)

With one plate (fig. 1)

SUMMARY

1. Activity of the specific alkaline phosphatase, 5-nucleotidase, is intense in the epithelium and secretion of the rattlesnake venom gland. Non-specific alkaline phosphatase activity is lacking.
2. Thyroid epithelium, the smooth muscle of great vessels, and (inconstantly) smooth muscle of abdominal hollow viscera show greater 5-nucleotidase than non-specific activity.
3. These findings confirm the specificity of 5-nucleotidase.

INTRODUCTION

THERE has been controversy concerning the existence of a number of specific alkaline phosphatases. Evidence is accumulating for the identity of a distinct and specific enzyme which liberates phosphate from 5-nucleotides (e.g. adenosine-5-phosphate, muscle adenylic acid, and inosine-5-phosphate, inosinic acid) but not from 3-nucleotides (adenosine-3-phosphate, yeast nucleic acid).

To this enzyme the name 5-nucleotidase was given by Reis (1934) who described its occurrence in extracts of heart muscle. In subsequent papers (1937 *a*, 1937 *b*) Reis reviewed the distribution of 5-nucleotidase, finding it in significant quantities in nervous tissues, thyroid, testicle, aorta and other vessels, lung, retina, and choroid. Species differences were noted. The rat contains the enzyme in nearly every tissue while the pigeon has demonstrable 5-nucleotidase activity only in the lungs.

Few histochemical studies of 5-nucleotidase have been reported. Gomori (1949), using muscle adenylic acid as substrate in his alkaline phosphatase technique, reported colouring of certain brain structures in the mouse and rat, spermatogenic elements in man, mouse, and rat, splenic follicles in man, and smooth muscle of visceral and blood-vessel walls. Newman and others (1950) demonstrated 5-nucleotidase activity in the media of the arteries in many organs and in smooth muscle of the walls of viscera. Elsewhere they found

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the activity with muscle adenylic acid as substrate was about the same as with other phosphates. Pearse and Reis (1952) recently reported that they were able to demonstrate histochemically 5-nucleotidase activity in those organs in which chemical estimations had previously indicated substantial activity of the enzyme. McManus, Lupton, and Harden (1952) presented a method for the histochemical demonstration of 5-nucleotidase (see below). Its occurrence in a number of human tissues was reported. The presence of 5-nucleotidase and the absence of alkaline phosphatase in obsolescent human renal glomeruli were indicated by McManus and Lupton (1952). McManus, Lupton, and Etheridge (1952) described features of 5-nucleotidase and other phosphatases studied histochemically in the aorta and other blood-vessels of man.

Because of the interesting distribution of this enzyme in the tissues of man and other mammals, and the reports of Gulland and Jackson (1938) and Zeller (1951) that an enzyme with specific 5-nucleotidase activity has been found in all snake-venoms thus far examined chemically, we have attempted its demonstration in the poison and other salivary glands of snakes and in other snake tissues.

MATERIALS AND METHODS

The method used in these studies was the McManus, Lupton, and Harden (1952) modification of Gomori's alkaline phosphatase technique. Tissues were fixed in chilled 80 per cent. alcohol and embedded in paraffin. Sections were incubated for 2 to 4 hours or overnight in Gomori's alkaline phosphatase stock solution at pH 8.5 to 8.8, lacking magnesium ion and containing muscle adenylic acid or inosinic acid as substrate. Corresponding sections were treated by the alkaline phosphatase method of Gomori, with glycerophosphate and hexosediphosphate as substrates. Precipitated phosphates were visualized by the formation of black cobalt sulphide. Tissues were obtained from garter snakes (*Thamnophis sirtalis sirtalis*), juvenile pilot black snakes (*Elaphis obsoleta obsoleta*), western diamond-backed rattlesnake (*Crotalus atrox*), and a few tissues from water-snakes (*Natrix sipedon*). Tissues examined included salivary glands, thyroid, adrenal, intestine, pancreas, spleen, liver, trachea, lung, heart, aorta, testis, ovary, kidney, brain, eye, and Harderian gland.

RESULTS

Black precipitates, indicative of enzyme activity, are marked in a number of snake tissues incubated with muscle adenylic acid. Nuclei and nucleoli are often well coloured. However, the intensity of coloration is usually proportional to cytoplasmic activity in the vicinity. In view of the findings of Martin and Jacoby (1949) and others, it is our feeling that this may represent adsorption of calcium phosphate and not intrinsic enzyme activity.

Precipitates are formed in cartilage, lung, the luminal borders of oral and intestinal mucosal cells, vas deferens epithelium, and liver and adrenal

musoid endothelium. In the kidney, activity in the proximal convoluted tubules is concentrated at the brush borders, while in the distal convoluted tubules it is associated with granules dispersed throughout the cytoplasm (fig. 1, H). However, there is also blackening in these areas with hexosediphosphate and glycerophosphate. Because activity is manifested towards all three phosphate substrates in these regions, the most that can be said is that an enzyme is present which is effective in hydrolysing 5-nucleotide. The evidence does not permit the conclusion that a specific enzyme for 5-nucleotides only is present.

The most striking proof of a specific 5-nucleotidase is obtained with poison glands of the rattlesnake, *Crotalus atrox*. The active poison gland is made up of branching tubules with wide lumina lined with a simple columnar epithelium. There is intense staining in both secretion and epithelium, with muscle adenylic acid as substrate (fig. 1, A). With glycerophosphate (fig. 1, B) there is almost no coloration. This is consistent with Zeller's finding that the venom of viperine snakes is rich in 5-nucleotidase but lacking in phosphomonoesterases.

In contrast, salivary glands of both poisonous and non-poisonous varieties show no greater activity with 5-nucleotides than with other substrates. In sections of the superior labial gland of the garter snake (fig. 1, G) the serous cells show phosphatase activity with both muscle adenylic acid and glycerophosphate. By contrast only the mucus-cells react positively with the PAS technique. The rattlesnake labial gland differs from that of the garter snake in having a greater preponderance of mucus-cells.

The smooth muscle of the blood-vessels and hollow viscera is another tissue in which there is a distinct difference in activity between the muscle adenylic acid and glycerophosphate preparations. The media of the large arteries near the heart is consistently darker with muscle adenylic acid (fig. 1, C) than with glycerophosphate (fig. 1, D), although differences in the degree of coloration are not always marked. As has also been noted in mammalian tissues by Gomori and in our laboratory, the coloration in visceral smooth muscle, although often intense, is inconstant and is frequently lacking. Thyroid epithelium also shows greater activity with 5-nucleotide (fig. 1, E) than with glycerophosphate (fig. 1, F).

Histochemically, our findings in the rattlesnake venom gland and the smooth muscle of blood-vessels and hollow viscera, taken in conjunction with work on mammalian tissues in our own and other laboratories, would seem to establish 5-nucleotidase as an enzyme separate from phosphomonoesterase and hexosediphosphatase.

This study was aided by a grant from the National Institutes of Health, United States Public Health Service.

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FIG. 1 (plate). A, C, E, and G (on the left), and H (on the right), are alkaline phosphatase preparations with muscle adenylic acid (MAA) as substrate according to the method of McManus and others; B, D, and F (on the right), glycerophosphate was used as substrate.

A and B, *Crotalus atrox*. Collapsed tubules of venom gland. 5-nucleotidase activity is intense in epithelium and venom coagulum. Non-specific phosphatase activity is absent. ($\times 67$.)

C and D, *Thamnophis sirtalis sirtalis*. Heart (above) and arterial trunk (below). Smooth muscle of the media of the arterial trunk shows greater activity towards 5-nucleotide than toward glycerophosphate. ($\times 67$.)

E and F, *Crotalus atrox*. Thyroid gland. Epithelial cells show greater 5-nucleotidase than non-specific phosphatase activity. ($\times 67$.)

G, *Thamnophis sirtalis sirtalis*. Section through upper lip. Phosphatase activity (substrate: MAA) is apparent in buccal mucosa (lower right) and epithelium of superior labial salivary gland (left) and its duct; absent in epidermis (upper right). ($\times 60$.)

H, *Elaphe obsoleta obsoleta*. Kidney. Alkaline phosphatase activity is intense in the brush borders of proximal convoluted tubule cells. Precipitate in distal convoluted tubules (e.g. the lower right of glomerulus) is associated with coarse granules throughout the cytoplasm. ($\times 233$.)

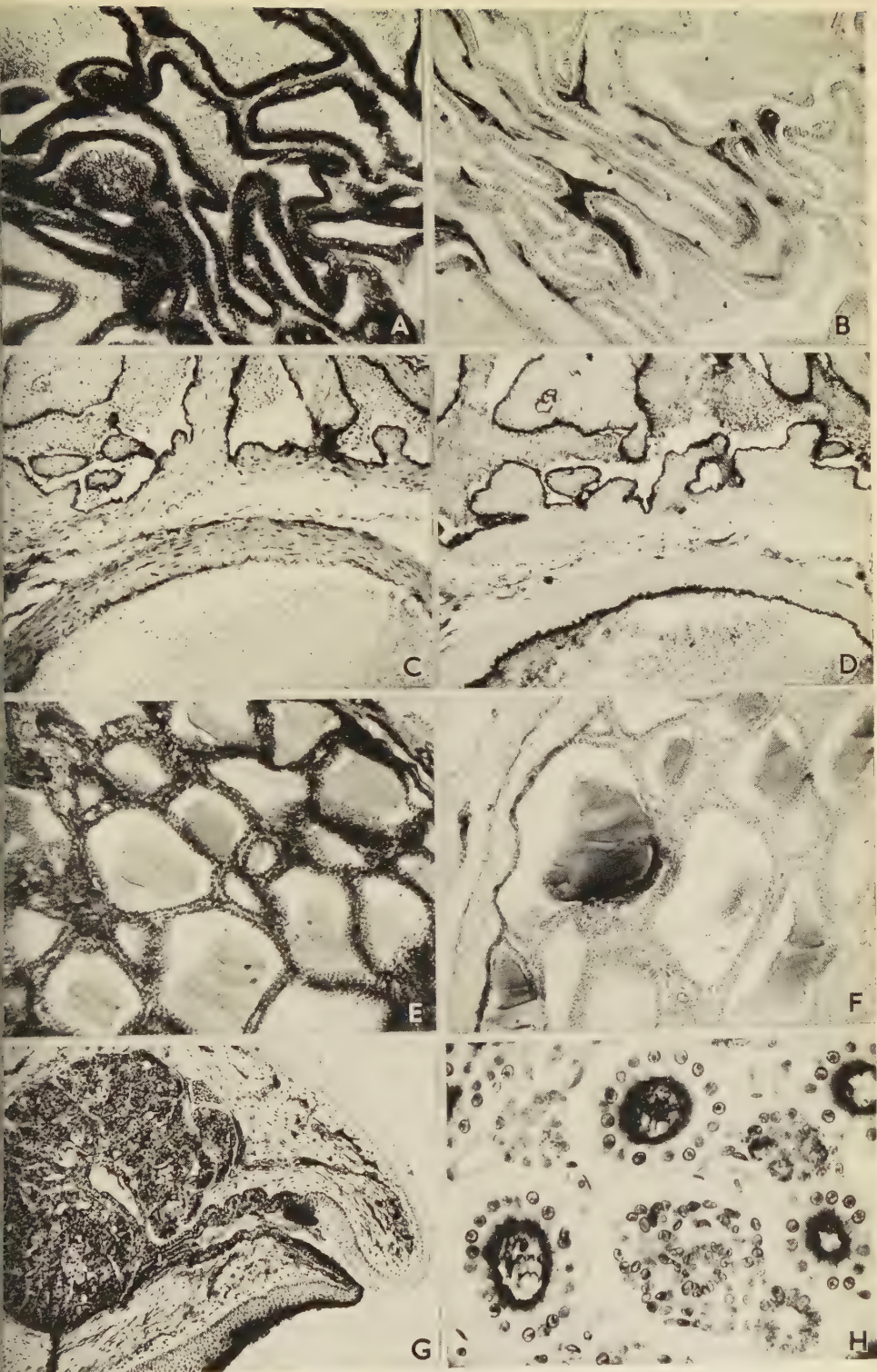


FIG. 1
D. E. BRAGDON and J. F. A. McMANUS

Phase Contrast and Electron Microscope Studies of the Appearance and Behaviour of the White Cells of Normal Human Blood

By JUNE MARCHANT

(From the Zoology Department, University of Birmingham)

With three plates (figs. 3-5)

SUMMARY

1. The phase-contrast microscope has been used to study form and movement of human leucocytes and blood platelets in three types of preparation; (a) warm sealed blood drops, (b) warm sealed films of leucocytes adhering to coverslips after incubation of blood drops at 37° C. for 30 minutes or more, and (c) warm sealed films of blood platelets obtained by similarly incubating drops of saline suspensions of these cells. Similar preparations to (b) and (c) were obtained on formvar films and studied in the electron microscope. Phagocytosis was studied in all the above preparations after adding suspensions of collodion particles or of *Staphylococcus albus*.
2. The neutrophils were the most versatile cells seen in the above preparations. The amoeboid forms were the most mobile and phagocytic. Another form showed rapidly waving processes which gradually spread into a flat membrane on the glass. Flattened immobile neutrophils were still capable of phagocytosis. Bacteria which adhered to the cell surface moved over it towards the thicker central region of the cell where ingestion occurred.
3. Eosinophils showed a mobile amoeboid form and flattened forms which were often very bizarre in shape. They did not phagocytose readily.
4. Monocytes showed slowly moving amoeboid forms or forms with ruffle-like membranes around the cell. They were phagocytic.
5. Lymphocytes were capable of amoeboid movement but did not flatten on glass and were not phagocytic.
6. Blood platelets showed dendritic and flattened forms. Bacteria or collodion particles adhered to the surfaces of the latter and travelled over them, clustering at their thicker centres.

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INTRODUCTION

DURING the past decade several authors have published studies of leucocytes and blood platelets, using the phase-contrast and electron microscopes. The flattening of leucocytes was studied with the phase-contrast microscope by Bessis and Bricka (1949 *a* and *b*), and their structure described by Bessis (1949*a*) and by Feissly and Lüdin (1949). These authors mentioned briefly a few observations on the movement of living leucocytes and Bessis described various degenerate changes in living leucocytes kept at room temperature. Feissly and Lüdin also described the structure of blood platelets.

Using the electron microscope Bessis (1949*b*) and Rebuck and Woods (1948) studied the ultra-structure of leucocytes. The fine structure so revealed included that of the chromatin of lymphocyte nuclei (Rebuck, 1949), the pseudopodia of leucocytes (Bernhard and his co-workers, 1950 *a* and *b*), and the granules (Bessis and Bricka, 1950 *a* and *b*). The flattening of leucocytes was described by Bessis and Bricka (1949*c*). Rebuck and Woods (1948) published the only electron microscope picture of phagocytosis by a leucocyte. Pictures showing the ultra-structure of blood platelet cytoplasm have been published by Bessis and Bricka (1948), Bessis and Burstein (1948), and by Bessis (1949*b*). Their structure and its changes during flattening have been described by Wolpers and Ruska (1939) and by Bessis (1949*b*).

In the work presented here the phase-contrast and electron microscopes have been used to study both the structure and the behaviour of normal human leucocytes and blood platelets. Special attention has been given to movement and phagocytosis, and a very simple method of obtaining phagocytosing leucocytes for electron microscopy was evolved.

. TECHNIQUES

Methods of concentrating leucocytes and platelets for study

The method used for concentrating leucocytes other than lymphocytes was that described by Wright and Colebrook (1921). A wax ring 1 cm. in diameter was made on a glass slide or coverslip. The cell thus obtained was filled with fresh finger-prick blood and placed in a Petri dish lined with moist filter paper. After incubation at 38° C. for 30 minutes or more, the blood clot was removed and the preparation washed free from red cells with normal saline. A film of leucocytes adhered to the glass in approximately the following proportions; neutrophils 80 per cent., eosinophils 10 per cent., monocytes 10 per cent. Lymphocytes were rarely present. When citrated blood was used, no adherence of leucocytes was obtained.

To obtain blood platelets, a few drops of blood were mixed in a wide tube with about 5 c.c. of citrate saline containing 0.6 per cent. dextran. The dextran accelerated the sedimentation of red cells (Minor and Burnett, 1948). When this was complete the supernatant fluid was pipetted off and the suspended platelets were centrifuged and washed with normal saline. A drop of the concentrated platelet suspension was then incubated in the same manner as the fresh blood (described above) for periods of up to 30 minutes. Examination of these preparations showed platelets in the process of flattening themselves against the glass surface.

Blood of group A₂, R₁r from a single normal human being was used in these studies.

Preparation of materials for phagocytosis

Collodion particles have previously been used in phagocytosis experiments (see Mudd and others (1934)). In my experiments suspensions of collodion particles were prepared in the following manner: a 1 per cent. solution of collodion in alcohol-ether was diluted 15 times with acetone and carefully poured on top of an equal volume of distilled water in a separating cylinder. The acetone mixed slowly with the water, so precipitating collodion particles which settled and were run off at the bottom. The suspension of particles was then dialysed in a collodion membrane against distilled water, or centrifuged and washed several times. The final suspension was made in normal saline.

Suspensions of *Staphylococcus albus* for phagocytosis were prepared by gently rubbing the surface of agar slope cultures with a glass rod and washing with a few c.c. of normal saline.

Ordinary light microscopy

Preparations fixed and stained by various techniques were observed in the ordinary light microscope to facilitate their interpretation when observed by phase-contrast microscopy.

Phase-contrast microscopy

A Cooke, Troughton, and Simms binocular phase-contrast microscope was used in observing living and fixed cells and platelets. A thin sheet of copper, bearing a heating element controlled by a rheostat potentiometer, could be fitted to the mechanical stage. Living preparations were placed over the hole in the plate.

Suitable preparations were obtained by placing a very small drop of blood or a film of leucocytes or platelets, together with bacterial or collodion particle suspensions when required, on a thin coverslip and carefully lowering on to it a slightly smaller coverslip. The slips were then sealed with paraffin wax and the preparation clipped over the hole in the warm stage of the microscope with the larger slip below. It was then possible to use the $\times 95$ oil-immersion objective.

Phase-contrast photographs were taken with a ciné outfit designed by

Walker and Davies of the Biophysics Research Unit, King's College, London. They were taken on Ilford extra-fine-grain panchromatic film with an exposure time of 2 seconds.

Electron microscopy

A solution of 0.5 per cent. formvar in dioxane was used to prepare formvar films on glass slides. When the film was dry a square about 2 cm. $\times$ 2 cm. was scored round with a sharp knife and floated off on to distilled water. A wax ring was made on this by dipping a piece of 1 cm.-bore glass tubing into hot paraffin wax and carefully touching it down on the formvar film. It was then refloated on to a slide and drained of excess water.

A dense population of leucocytes or platelets was obtained on the film by Wright and Colebrook's (1921) technique. In studies on phagocytosis the suspension of collodion particles, or of bacteria and fresh human serum, was then added and the preparation reincubated. In some cases the particles for phagocytosis were mixed with whole blood at the beginning of incubation.

After washing in warm saline the preparation was fixed. Many simple and mixed fixatives were tried, but the most life-like appearance was given by osmium tetroxide. Fixation for 5 to 10 minutes in a fresh 2 per cent. solution, or in the vapour, was used in this study. Occasionally absolute alcohol was used. The preparation was then washed in distilled water and the formvar film carefully stripped from the slide. The wax ring caused the film to float so that it was easily mounted on the specimen screen used in the microscope. Preparations were stored in a desiccator and shadowed with palladium gold at an angle of about 45° .

The electron microscope used was a Metropolitan-Vickers E.M.3. Photographs were taken at 75 kv.

RESULTS

Comparison of the information yielded by the microscopical methods used

The study of living leucocytes by ordinary light microscopy was very difficult on account of their extreme transparency, which makes their contents almost or quite indistinguishable from one another. In order to obtain information about the cell constituents by this method it was necessary to resort to leucocytes which had been fixed and stained. The disadvantage of this was that one could no longer study living processes such as movement or phagocytosis as they occurred in any particular cell. Phase-contrast microscopy has overcome these difficulties. The particles in living leucocytes which were invisible by ordinary light were made darker or lighter than their background and their movements could be followed readily. Phase contrast is best used on very thin or transparent objects which have little phase difference. It is difficult to film rapidly with phase-contrast illumination since long exposures are required.

Both the above forms of microscopy suffer from the fact that resolution is limited by the wave-length of visible light. The electron microscope enables

far higher resolution to be obtained by using a beam of electrons instead of light-rays. Its chief drawbacks in a study such as this were that the cells had to be extremely flattened and obtained on delicate films to allow penetration by the electron beam. The cells had to be fixed and vacuum-dried and these processes produce artifacts. However, one simple fixative of the many which were tried gave a fairly life-like picture of the leucocytes. This was osmium tetroxide. It preserved the homogeneity of the cytoplasm, avoiding the sponge-like appearance produced by protein precipitants. Little shrinkage appeared to be caused by it in the cell as a whole, but collodion particles and sometimes eosinophil granules had a shrunken appearance.

Morphology and movement of leucocytes and platelets

In the two types of preparation studied (sealed blood drops and sealed films of leucocytes obtained by Wright and Colebrook's technique) the same type of leucocyte assumed a number of quite distinct forms. The greatest variety of form was seen amongst the *neutrophils*. In sealed blood drops kept at 38° C. and viewed by phase-contrast microscopy the neutrophil immediately began to flatten from the spherical form of 10 to 12 μ in diameter to a roughly pear-shaped form and began to move, broad end first. The most typical size and shape of these fresh, active neutrophils was that seen in fig. 3, A-D, perhaps 20 to 25 μ long, 10 to 15 μ in width at the broad end, narrowing slightly for 15 to 20 μ and then more suddenly to leave a short, narrow tail. Sometimes there was no tail (fig. 3, D), but at other times, particularly as the preparation aged, it was 20 μ or more long and occasionally divided into a number of fine cytoplasmic processes (filopodia).

The fresh active neutrophil was capable of moving roughly 30 μ per minute at 38° C. At the broad front end of the cell was a hyaloplasmic border up to 5 μ in depth which advanced in a continuous flow. Behind this was a region of cytoplasm containing many neutrophil granules, those in the central zone showing a fairly continuous Brownian movement, combined with a forward and outward streaming as the cell flowed forward (fig. 1). The nucleus, which comprised 1-5 unequal lobes, occupied most of the main body of the cell and was surrounded by more granules. The tail was almost or quite free from granules and was a relatively permanent structure. As the front end of the neutrophil flowed forward, the tail moved more slowly, with the result that the whole cell became somewhat elongated, the nucleus often being considerably deformed in the process. The granules streamed slowly up the centre of the cell, vibrated at the front end and then moved slowly out towards each side, where they remained more or less stationary. At intervals of about 20 seconds to 2 or 3 minutes, according to the rate of movement of the cell, the region just in front of the tail appeared to become more fluid. Nucleus and granules streamed rapidly to the front region, causing the main cell body to become more rounded, the tail being drawn up later. Tail filopodia were quite stiff structures, as could be seen when a granulocyte changed direction and they were swung through the medium.

When a neutrophil came up against an obstacle such as a platelet clump in its path, it either pushed its way round one side of it or it would pass round both sides of it and become almost divided into two. The halves would continue to advance until they were perhaps 100μ apart, remaining attached to one another by a slender thread of cytoplasm (fig. 3, E). The nuclear lobes would be distributed between the two halves, remaining connected by a very fine strand of nuclear substance. Eventually one half would continue its

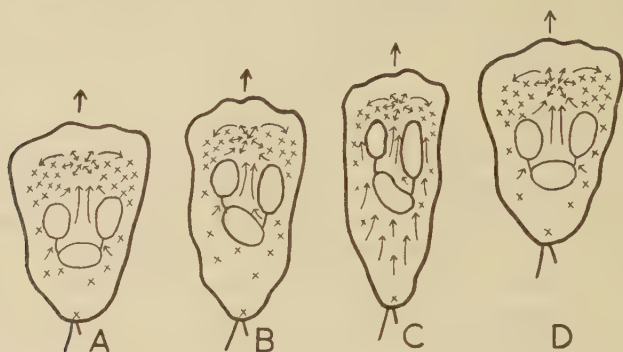


FIG. 1. Typical cytoplasmic currents in a neutrophil resulting in movement forward. Arrows represent granules in motion. Their length indicates speed of motion. Crosses represent sites of stationary granules. A, B, C, and D are successive stages in movement.

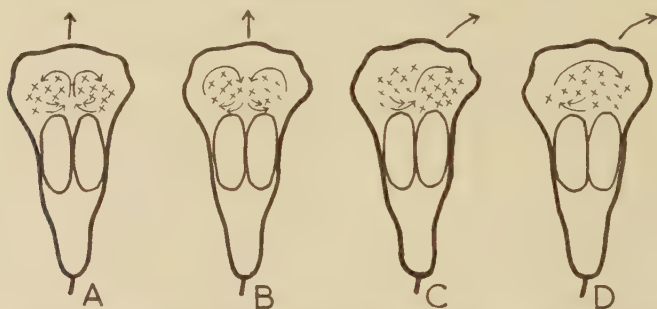


FIG. 2. Cytoplasmic currents seen in eosinophils resulting in various movements.

advance, whilst the hyaloplasmic tip of the other became invaded by granules showing very vigorous Brownian movement. This half would then stream rapidly back into the other half and the whole cell might subsequently move in the normal manner.

In ageing blood drop preparations neutrophils often put out numerous pseudopodia which were shortly withdrawn again into the main cell body after showing Brownian movement of the granules up to the cytoplasmic edge. After 40 minutes' or so incubation by Wright's technique, motile, pear-shaped forms were sometimes obtained which were rarely smooth in outline like those seen in sealed blood drops, but had their cytoplasmic surfaces irregularly thrown up into folds.

Phase contrast observations also revealed another form of neutrophil in Wright's preparations incubated for an hour or more and a few were sometimes seen in sealed blood drops. The cell body had taken on a flattened oval or circular shape about 22 to 25 μ in diameter, often surrounded by numerous processes (fig. 3, L-O). The flattest forms of all were obtained on the lower surface of a coverslip when this had been placed on top of a blood filled wax cell to form a roof in Wright and Colebrook's technique. These flattened neutrophils were capable of changing their outlines slightly and of phagocytosing foreign particles which came into contact with them (fig. 3, L-O). Granules within the cell showed some slight rearrangements and sometimes vigorous Brownian movement in small localized areas of the cytoplasm.

The flattened neutrophils were the most suitable forms for electron microscopy and the details revealed by it will be described below. There was great variation in the cell outline. Sometimes it was fairly smooth or gently undulating, sometimes surrounded by numerous processes which might be long or short, fine or stout, branched or unbranched. Processes of one neutrophil were apparently capable of fusing with those of another (fig. 4, A). Often something of a pear-shaped outline was retained by these flattened neutrophils. In such cases there would be a blunt end with undulating outline, sometimes having the appearance of two overlapping folds of cytoplasm, and a tail end bearing filopodia. The hyaloplasmic border of the cell varied from 1 to 5 μ in width. Surrounding the central nuclear lobes was an area of granular cytoplasm 2 to 6 μ in width. The granules were round, about 300 to 400 in number, and varied in diameter from 0.6 μ down to 0.1 μ , most being about 0.3 μ . Among them it was sometimes possible to distinguish some 20 to 30 rod-shaped mitochondria 0.1 μ to 0.3 μ in width and 0.6 μ to 1.0 μ in length.

With the phase contrast microscope it was occasionally possible to find a dendritic form of neutrophil in a freshly sealed Wright's preparation. In these the nucleus and granular cytoplasm were concentrated into a roughly spherical shape, whilst the surrounding hyaloplasm was drawn out into narrow, unbranched dendrites up to about 10 μ in length and 0.6 μ in diameter, which waved in the surrounding medium (fig. 3, H). These dendrites grew rapidly to a width of 1.0 to 1.5 μ and ceased to wave. Then from their bases they began to broaden and to merge with one another, with the result that a hyaloplasmic membrane gradually spread outwards around the cell producing a flattened neutrophil (fig. 3, I-K).

Flattened neutrophils obtained after Wright's technique were sometimes seen to change into 'prickle cells' by a sudden contraction and rounding up of the main cell body, whilst the hyaloplasmic processes remained fixed to the substratum, becoming drawn out into long straight filopodia (fig. 3, P).

Yet another form of neutrophil was seen in some ageing Wright's preparations. It had a dense, central perinuclear region bearing short hyaloplasmic processes or a membrane; but the hyaloplasm in contact with the substratum was spread out farther. Thus there were overlying hyaloplasmic membranes or processes. Some of the cells studied by electron microscopy were

believed to be neutrophils of this form, and they were capable of phagocytosis (fig. 4, F).

The *eosinophil* was in many ways similar in behaviour to the neutrophil. It was about the same size and in sealed blood drops viewed by phase contrast illumination at 38° C. it assumed a similar pear-shaped, motile form, though generally it showed a smoother outline. The nucleus consisted almost invariably of two equal lobes, oval in shape (occasionally of one or of three unequal ones) which occupied a position side by side towards the narrow end of the cell. Almost all of the large eosinophil granules were concentrated towards the broad front end of the cell in front of the nucleus, where there was a narrow hyaloplasmic border 1 to 2μ in depth. The hyaloplasmic tail region was often furnished with filopodia of varying lengths and complexity.

Movement of the pear-shaped eosinophil was similar to that of the neutrophil, but in most cases a little slower. Some slight Brownian movement of granules in the forward central zone was distinguished, but streaming movements were often rapid and clearly seen. There was the typical fountain-shaped current of granules moving up the centre of the cell to the front and out towards the sides where they came to rest as described in the neutrophils (fig. 2, A). There were at other times currents which were the reverse of the fountain. Granules from the sides near the front end travelled swiftly forwards and then moved in towards the centre where they came to rest until carried backwards and outwards to the sides as other granules piled up in front (fig. 2, B). Both of these types of currents resulted in movement straight ahead. Asymmetrical currents as seen in fig. 2, C and D accompanied a turning of the cell to one side.

In Wright's preparations seen by phase contrast illumination a few flattened eosinophils were found with central nucleus surrounded by granules and an outer border of hyaloplasm (fig. 3, R). They were often somewhat elongated with the nuclear lobes placed end to end. Most of the granules were clustered around the nucleus. The hyaloplasmic border of the cell was of variable width and extended into processes of many shapes and sizes, some of which contained isolated granules.

The flattened eosinophils were the cells most easily recognizable in the electron microscope by reason of their large, opaque granules. These num-

FIG. 3. Phase-contrast photographs of living leucocytes. A-G in sealed blood drops, H-T in sealed Wright's preparations. A-D, a lymphocyte and a moving neutrophil taken at intervals of 10 seconds. Note the sudden acceleration of the neutrophil from C to D. Three blood platelets 'p' were also present. E, an elongated neutrophil phagocytosing a bacterium. 'Digestive' vacuoles containing several bacteria were already present. F and G, phagocytosis of two bacteria by a neutrophil and formation of a 'digestive' vacuole in 20 seconds. H-K, a dendritic neutrophil in various stages of flattening. This took 7 minutes. L-O, a flattened neutrophil phagocytosing a bacterium 'b'. Six minutes elapsed between L and O. P, the same neutrophil as above taken 30 minutes later when it had become transformed into a 'prickle' cell. Q, a nearly flattened dendritic neutrophil. R, a bizarre flattened eosinophil. S and T, a monocyte showing waving of the ruffle-like membrane, 40 seconds between exposures.

bered about 100 to 150 and were usually round, oval, or shaped like short rods. They varied in size from 0.3 to 1.7μ , but the majority were about 0.6μ in diameter (fig. 5, A). Some eosinophils also showed a few much smaller, less opaque particles down to about 0.01μ in diameter. In some electron microscope pictures there appeared to be fine strands of black material connecting some of the granules to one another. The same effect was observed when collodion particles were fixed in osmium and looked at in the electron microscope. Fig. 4, D is an electron microscope photograph of an eosinophil which has retained its pear shape.

Basiphils were not usually seen, being very few in number, but it was considered that fig. 4, C might be an electron microscope picture of one. It showed a large, bilobed nucleus and a small number of granules which were not distinct and appeared to be far fewer and more irregular in size than those of typical eosinophils.

In sealed blood drops and in Wright's preparations *monocytes* were usually extremely flattened and indistinguishable from their background by phase contrast illumination. The nucleus was round, oval, indented or kidney-shaped, measuring 7 to 11μ in diameter. It was more or less centrally placed in the cytoplasm which measured 17 to 21μ in diameter. The cytoplasm was peppered with fine granules except for an outer border 2μ in width. The cell shape was roughly circular or oval, the outline smooth and usually gently undulating. These flattened monocytes showed a slow change in shape of nucleus and cytoplasm with little change in position of the cell.

In the electron microscope the granules in flattened monocytes were seen to number up to about 120 (fig. 4, B). Up to 20 of these were usually distinct and measured 0.3 to 0.6μ in diameter, the rest being smaller, less opaque and difficult to distinguish. Up to 30 were very pale rod-shaped mitochondria measuring 0.4 to 1.5μ in length and 0.1 to 0.2μ in diameter.

In fresh Wright's preparations the monocytic cytoplasm seen by phase contrast illumination was not always spread out flat in one layer on the substratum. Sometimes the cell appeared fairly spherical with one folded membrane, or possibly several independent membranes thrown out in different planes around it. Such membranes were described by Goodrich (1919) in leucocytes of invertebrates. Waves passed fairly rapidly and regularly around these membranes (fig. 3, S and T). At other times the cell was more flattened and the overlying membranes irregularly shaped. Sometimes the membranes were folded or ridged with the ridges elongated into fine processes. The membranes

FIG. 4. Electron microscope photographs of osmium-fixed leucocytes after Wright's technique. A, a flattened neutrophil capturing 4 bacteria 'b'. Note apparent fusion of two processes with those of a neighbouring cell. B, a flattened monocyte capturing bacteria 'b'. C, a basiphil? or eosinophil. D, an eosinophil. E, a neutrophil phagocytosing collodion particles. The cell contained many particles but was continuing to phagocytose. Various stages in the process may be seen. F, a double membraned neutrophil showing a 'digestive' vacuole containing 4 bacteria. Note the rounded cell body surrounded by a hyaloplasmic membrane which is overlain by cytoplasmic processes at a higher level.

were capable of extending and withdrawing slowly so that there was some change in shape of the cell. Such movement was probably similar to that of the undulating membrane of monocytes in tissue culture described by Carrel in 1926.

In Wright's preparations *lymphocytes* almost always failed to adhere to the substratum and they were subsequently washed away with the red cells. Thus they were not obtained for electron microscopy. In sealed blood drops they were often rounded and immobile, but when the glass surfaces were closely pressed together they often showed great activity.

The *large lymphocyte* was about 12 to 15 μ in diameter and tended to show an elongated or pear-shaped form. At the broader end was a border of cytoplasm, up to 3 μ in depth, and this was followed by the nucleus which measured 8 or 9 μ in diameter. The bulk of the cytoplasm lay behind this and it tapered slightly towards the end.

The process of lymphocyte movement was similar to that of the granulocytes and the cell was capable of moving at over 20 μ per minute. The broad end of the cell flowed forward more rapidly than the narrow end, so that the cell and its nucleus became elongated. Then at intervals the lymphocyte would draw up its tail and the nucleus would round up again. The deformability of the nucleus was very striking.

Many of the *small lymphocytes* appeared as rounded non-motile cells with a smooth outline, measuring 8 or 9 μ in diameter, thus occupying most of the cell volume. The cytoplasm was scanty and clear, but 20 to 30 grey, rod-shaped mitochondria were usually distinguishable by phase contrast microscopy, mostly clustered at one side of the nucleus. In about half of these cells 1 or 2 large, dark granules were also distinguishable, with occasionally a few small ones as well.

Some of the small lymphocytes were capable of directional movement at a rate of about 10 to 15 μ per minute in a similar manner to the large lymphocyte, though the nucleus was not so deformable. It was capable of becoming indented, heart or dumb-bell shaped. The mitochondria and granules usually followed after the nucleus during movement. Other small lymphocytes showed a very small degree of movement (fig. 3, A-D).

Blood platelets in freshly sealed blood drops appeared either singly or in clumps as dark grey bodies about 2 μ in diameter with a lighter, irregular periphery by phase contrast illumination (fig. 3, A, 'p'). Occasionally some were seen shaped like tadpoles. On incubation the single platelets and the outer ones of a clump appeared to swell and become a lighter grey. Eventually they became refractile circles about 3 to 7 μ in diameter, or even larger. Some then became free from their neighbours in a clump and floated away in the serum. Often a number of minute granules in rapid Brownian motion were distinguishable within them.

Platelets separated from citrated blood were studied by electron microscopy. A series of preparations incubated for gradually increasing time intervals revealed the method of flattening against the substratum. At first the platelets

appeared with a dense, irregularly shaped centre of 1 to 2 μ in diameter bearing numerous finger-like projections 4 μ or more in length (fig. 5, c, 'd'). These were the dendritic forms described by Bessis (1949b). Later forms showed dendrites with widened bases which joined to form a web or membrane (fig. 5, b). Finally, a flattened form was reached which measured 4 to 6 μ in diameter and was often circular (fig. 5, c). Some giants of up to 30 μ in diameter were believed to be the result of fusion of several platelets (fig. 5, g). Similar preparations of unfixed platelets and osmium fixed platelets showed identical forms when studied by phase contrast microscopy. The cytoplasm of several platelets showed holes (fig. 5, d and g). These were probably formed by the fusion of the tips of dendrites as they expanded. The lower half of the platelet in fig. 5, e shows how this might occur. The central region, occupying about one third of the platelet diameter, was the densest part, but it sometimes contained small vacuoles (fig. 5, g). The main bulk of the cytoplasm was less opaque, often showing denser ribs running radially to the tips of any remaining dendrites. Some platelets revealed cytoplasmic fibres running radially and merging with other fibres running parallel to the border (fig. 5, d). Many appeared to be non-granular, but a few had up to 100 round granules 0.1 to 0.3 μ in diameter distributed throughout the peripheral cytoplasm (fig. 5, c and g). In others only the dense central region of the platelet had a granular appearance.

Phagocytosis by leucocytes

Phagocytosis was studied in both sealed blood drops and in Wright's preparations by phase contrast and electron microscopy. The presence of fresh serum, in practice human serum, was required for phagocytosis of the bacteria used, but not for collodion particles. The latter tended to clump together, but the former moved about singly or in small groups.

By far the most actively phagocytic cell seen in the sealed blood drops by phase contrast microscopy was the *neutrophil*. The advancing hyaloplasmic edge of the fresh active forms would come into contact with bacteria or particles either singly or in groups and after a few seconds the hyaloplasm would flow round and over them to engulf them completely. The ingested material then traversed the hyaloplasm and entered the granular perinuclear zone of the cytoplasm where, if it was a bacterium, a digestive vacuole was formed round it in a few moments (fig. 3, f and g). When other digestive vacuoles were already present the newly entered bacterium sometimes travelled up to one of these and burst into it, joining the other bacteria there in Brownian motion. Several vacuoles formed around individual bacteria sometimes coalesced to form larger ones, so that a neutrophil might come to possess a few enormous vacuoles, each containing many bacteria. No digestive vacuoles were formed around collodion particles.

The whole process of phagocytosis up to the beginning of vacuole formation took sometimes as little as 1 minute, but often 3 or 4 minutes or longer. A fast moving neutrophil was capable of performing a separate act of phago-

cytosis every minute, or more frequently. These neutrophils could ingest a large clump of 20 to 30 collodion particles all at once by slowly flowing around it. This might take as much as an hour to complete, the cell being then so full as to be incapable of further movement.

Fig. 3, E shows phagocytosis by an elongated neutrophil. Sometimes, as in this case, a little tongue of hyaloplasm was thrown out which flowed round the bacterium.

Flattened neutrophils in Wright's preparations were also capable of phagocytosis in spite of their immobility. When a bacterium contacted a part of the cell surface which had a smooth outline there was a slight local outflowing of hyaloplasm to engulf the bacterium. If it contacted the tip of a short stout process the bacterium might be engulfed there and then pass along the process towards the perinuclear zone. If the process was a slender filopodium, the bacterium was sometimes engulfed by the tip and then it travelled along the process, causing local swelling on the journey, or the filopodium was retracted slowly into the cell. Frequently a bacterium did not appear to be engulfed when it contacted a filopodium, but it passed centrally along the filopodial surface at about 3 to 10μ per minute and then entered the hyaloplasm (fig. 3, L-O), sometimes causing neighbouring filopodia to be pushed aside through an angle of perhaps 20° . If a small group of bacteria were involved usually only one of them would be in contact with the filopodium, the others being towed along until the main cell body was reached. Ingestion was effected either by the bacterium merely sinking into the cytoplasm, or by the outflowing of a small tongue of hyaloplasm which wrapped round it.

Electron microscope pictures show cells fixed in various stages of the act of phagocytosis. Ingested collodion particles were seen within the cells as grey, circular bodies measuring 0.2 to 2.0μ or more in diameter with a darker border. When not ingested they usually underwent shrinkage, appearing as dense, black bodies with an irregular surface and with bridges between them. The shadows thrown by them were often wider than the particles themselves. Fig. 4, E is a neutrophil showing various stages in phagocytosis of collodion particles. The bacteria used (*Staphylococcus albus*) appeared as circular or rod-shaped black bodies 0.4 to 0.8μ in diameter.

It was difficult to study phagocytosis by *eosinophils* in the phase contrast and electron microscopes as it was often impossible to distinguish ingested material from eosinophil granules. In fixed and stained specimens

FIG. 5. Electron microscope pictures of a leucocyte and of blood platelets, all osmium-fixed except B, which was fixed in absolute alcohol. A, an eosinophil. B, 2 platelets, one nearly flattened, the other transitional from the dendritic phase. C, 3 platelets. A dendritic one lay on top of a completely flattened one which was granular. D, 3 platelets incubated 15 minutes after adding collodion particles. The large platelet showed cytoplasmic fibres, two large holes and clusters of collodion particles at the centre of the cell. E, a platelet incubated for 1 minute after adding collodion particles. They are seen near the edge of the cell, but are not clustered at the centre. F, 2 platelets from the same preparation as D. One had an unusual frilled appearance. G, 3 more platelets from the same preparation as D and F. One was a giant with granular cytoplasm and a thicker centre which had a vacuolar appearance.

seen by ordinary light microscopy it was seen that they were only feebly phagocytic.

The *monocytes*, on the other hand, were more phagocytic than the eosinophils, though less so than the neutrophils. They accomplished this by enveloping bacteria in the folds of their undulating membranes, or by merely sliding over adherent bacteria (fig. 4, B).

Bacteria often appeared to adhere to the surfaces of *lymphocytes* and in the case of cells believed to be large lymphocytes some of them seemed to have been ingested, though this was difficult to determine.

When the bacteria or collodion particles were washed from a preparation of leucocytes after a period of phagocytosis, it was found that within 5 minutes all ingested ones had reached the perinuclear granular zones of the cells.

Studies of platelets with particles

When suspensions of *blood platelets* were mixed with collodion particles, or bacteria and serum, the platelets did not flatten out on the substratum. However, if the platelets were allowed to flatten first and the bacterial or particle suspension was added later, some very interesting results were obtained. After 10 to 15 minutes incubation with collodion particles the electron microscope showed the platelets to have collected about 20 or more particles at their thickened centres, with a small number towards the periphery (fig. 5, D and E). Bacteria were not captured so readily. Collodion particles at the centres of the platelets had the shrunken, black appearance with connecting bridges and not the smooth, circular, grey appearance characteristic of particles well inside leucocytes. The adhesive forces between the particles or bacteria and the platelet were strong enough to withstand vigorous washing with a pipette.

When a collodion suspension was added to flattened platelets for only one minute before fixation all the particles were found towards the periphery of the platelet and none in the centre. The nearest particle to the centre was 2 or 3 μ from the periphery (fig. 5, E). Particles at the periphery were adherent to any part of the cell outline, often to the tips of the remaining dendrites.

DISCUSSION

The electron microscope techniques described in this work had the advantage of being simple to carry out and gave preparations comparable with those obtained by other workers who used much larger quantities of blood containing anticoagulants.

The extreme diversity of form seen amongst living leucocytes in the preparations studied in this work is, I believe, dependent chiefly on the fluidity of the medium and on the presence or absence of a solid substratum. When being swept along in the blood stream under pressure, leucocytes probably remain spherical in shape. When suspended in static fluid such as serum or anticoagulated shed blood the granulocytes may take on the dendritic form seen in fig. 3, H with lashing dendrites all round. Dendritic forms were described by Bessis and Bricka (1949a) in preparations made from anticoagulated

blood and Hope Simpson (1942) has described pus cells (granulocytes) covered with a coat of vigorously waving tentacles each 2μ in length. The dendritic neutrophil photographed in fig. 3, H was found in a Wright's preparation which had just been set up. Such forms were not found in older preparations, so presumably they were adapted to their environment immediately before the preparation was made. This environment would probably be serum exuded on the surface of the slide from the shrinking clot of blood above.

In this static fluid environment the form of monocyte equivalent to the dendritic form of neutrophil seemed to be that seen in fig. 3, S and T, surrounded by a rapidly waving membrane. Goodrich (1919) found that when invertebrate leucocytes were floating freely in a hanging drop their undulating membranes were motile in a similar way. One may assume that the lashing of dendrites or rapid waving of an undulating membrane might result in active movement of the leucocyte through the fluid medium.

I have found no evidence from my own preparations or in the literature of previous authors to suggest that lymphocytes have an analogous form which might be capable of movement through a static fluid medium.

When the dendritic neutrophils in Wright's preparations came into contact with a flat surface such as a glass slide they spread themselves out as shown in fig. 3, H-K. Bessis and Bricka (1949a) have also described various stages in the flattening of these dendritic forms. Conversion of flattened neutrophils into 'prickle' cells as described by Sabin (1923) was also noted (fig. 3, O and P). Similarly, monocytes tend to spread themselves out as an elongated film on a glass surface and the rapid mobility of their membranes slows considerably. Goodrich (1919) and Fauré-Frémiet (1929) observed similar flattening of invertebrate leucocytes on glass. They attributed the phenomenon to surface tension forces. In the process of flattening the membrane sometimes appears to become folded and by ordinary light only these folds are visible and they have the appearance of flagellate pseudopodia. The existence of the membrane between them was first recognized by Carrel in 1926.

In my preparations I have rarely seen anything resembling the degenerative changes seen in leucocytes by Bessis (1949a). I believe that the formation of a vacuole completely surrounding the nucleus, which he described, and the phenomenon of surface blistering or potocytosis, also described by Zollinger (1948), occurred only in cells which had been damaged mechanically.

When a spherical granulocyte or lymphocyte from a fresh drop of blood is placed between a warm slide and coverslip, rather than spreading itself out like the dendritic cells in Wright's preparations it may assume a polarity and amoeboid movement ensue. The movement is similar to that of the monopodal *Amoeba proteus* described by Mast in 1926. The theories of amoeboid movement are reviewed by de Bruyn (1946). At present the elastic tension of the superficial plasmagel layer is thought to provide the locomotor force. The superficial cytoplasm is thought to be in the gelled state, the gelled layer being thicker at the tail end and thinnest towards the anterior end of the cell, while

The rest of the cytoplasm is in the sol state. The gelated cytoplasm forces the fluid cytoplasm forward by its contraction. The plasmagel layer extends forwards by gelation of plasmasol at the sides of the anterior end of the cell, and continuous solation occurs near the posterior end of the cell.

Lewis (1939) described constriction rings during the movement of lymphocytes and granulocytes in tissue culture, and ascribed them to a thickening of the plasmagel in an equatorial band which remained fixed in relation to the cell environment until all the cytoplasm except part of the tail had passed through it. De Bruyn (1946) on the other hand considered they were caused by external factors in the medium such as fibrin strands or irregularities in the glass surface. In my preparations it appeared that the tail end of the cell often did not advance so fast as the anterior end so that the leucocyte gradually became elongated as shown in fig. 1, A-C. Suddenly there was a release of the tail which was rapidly drawn up and the cell assumed a more rounded form. It seemed as though the elastic tension of the plasmagel had suddenly overcome some resistance such as the adherence of the tail to the substrate. The regularity of contraction in any particular cell would seem to indicate that it was part of the regular mechanism of the movement rather than a contingent effect. If it were due to irregularities in the medium one would expect to see cells with more than one constriction ring at times, but I have not observed this.

I cannot offer support to Mast's theory that the plasmalemma executes a rolling movement during cell locomotion. Particles contacted by amoeboid neutrophils were either phagocytosed or ignored and they did not adhere to or travel forwards over the cell surface as in the *Amoeba* described by Schaeffer (1920). However, bacteria sometimes adhered to lymphocytes and in these cells they tended to collect at the posterior end of the cell and were not seen to travel forwards over the upper cell surface.

Blood platelets showed several features in common with leucocytes. When suspended in saline they assumed a dendritic form, but the dendrites did not appear to be capable of movement like those of neutrophils. On contacting a flat surface the platelets would spread out by a gradual widening of the dendrites from their bases towards their tips, just as in the case of neutrophils. The flattened forms were immobile. No amoeboid forms of platelets were seen. Other features common to platelets and leucocytes will be discussed later.

Since the work of Mudd and his collaborators in the early 1930's, little work has been reported on the mechanism of phagocytosis. The process falls naturally into three parts. Firstly, there is the approximation of particle and phagocyte, implying mobility on the part of one or other or both and ending in their contact. Secondly, there must be adhesion of cell and particle and, thirdly, the actual ingestion of the particle which is affected by the viscosity and elasticity of the cytoplasm.

When cells are able to move about their chances of contact with particles are increased. Leucocytes in their amoeboid form were capable of rapid move-

ment and therefore able to make frequent contacts with particles. In the case of flattened immobile leucocytes in the preparations studied, contacts with particles were made by their sinking through the suspending medium or by the Brownian movement of the particles themselves.

The adhesive stage of phagocytosis was not clearly seen in the amoeboid neutrophils which ingested them almost immediately on contact. However, it was seen in many other leucocytes. Amoeboid lymphocytes were seen with bacteria adhering to their tails. Particles adhered to the surfaces of flattened immobile neutrophils and platelets. They were then moved slowly over the plasmalemma towards the central region of the cell without any visible accompanying movement on the part of the cell as a whole. In the case of the neutrophils ingestion usually occurred when the particles reached the thicker granular region of the cell, but in the thinner platelets particles remained clustered at the centre of the flattened surface. The forces which cause adhesion of cell and particle are probably akin to those concerned in agglutination phenomena. Movement of particles towards the centre of a flattened cell surface probably results in a state of equilibrium between the forces participating in the adhesion.

The final ingestive phase of phagocytosis can only be accomplished when the cell or part of it is in a relatively fluid state so that the plasmalemma is able to make use of its elastic properties. An amoeboid neutrophil is in such a state and behaves rather like a fluid droplet, spreading as a result of surface tension forces.

In conclusion we may say that leucocytes and blood platelets are extremely versatile cells capable of adapting themselves to different environments in order to perform their cellular functions of movement and phagocytosis. The different leucocyte types participate in these processes to different extents, the neutrophil being the most active under the conditions of my experiments.

I should like to express my thanks to Mr. C. C. Newton of the Electron Physics Department for taking the electron microscope photographs and for many helpful suggestions; also to Professor J. T. Randall, F.R.S., of the Biophysics Research Unit, King's College, London, for allowing me to use the phase-contrast ciné microscope. Professor P. B. Medawar, F.R.S. initiated and encouraged the work.

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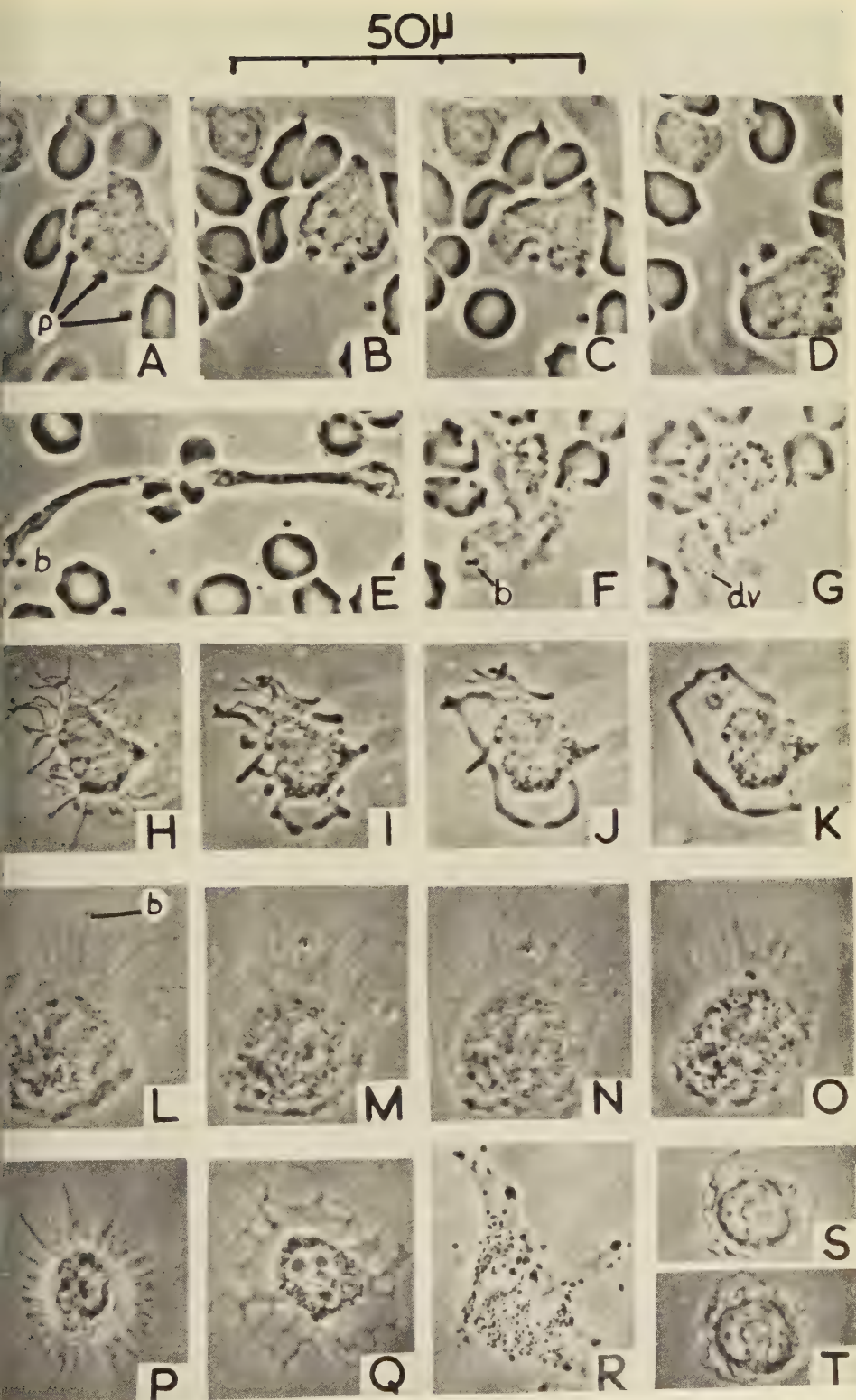


FIG. 3
J. MARCHANTII



FIG. 4
J. MARCHANT

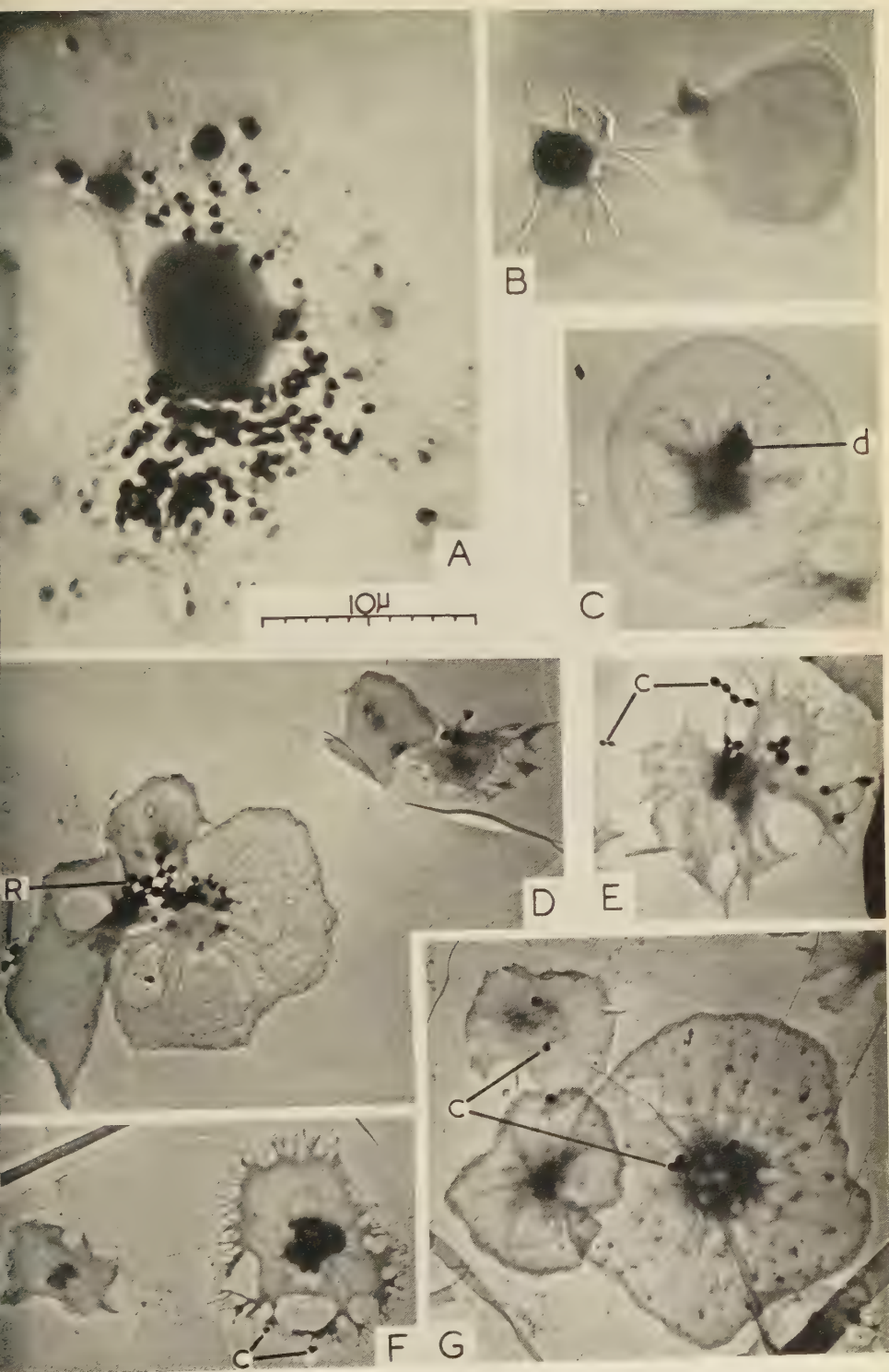


FIG. 5
J. MARCHANT

The Cytoplasmic Cytology of Sarcoma 180

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With four plates (figs. 1-4)

SUMMARY

Typical sarcoma 180 cells present a large spherical lipoid or Golgi zone adjacent to the nucleus within which discrete, homogeneous and vesicular, Golgi bodies (lipochondria) are distributed. No Golgi network can be detected in the living cell, but such networks may be produced artificially by means of hypertonic solutions and by shrinking vitally stained cells. All of the cytoplasm, except the lipoid zone, is strongly basophil owing to the presence of ribonucleic acid. Mitochondria, mostly rod shaped, form a halo of variable width surrounding the lipoid zone except adjacent to the nucleus. These lie partly within the ribonucleic acid containing cytoplasm. Tests for glycogen, alkaline, and acid phosphatase, and for lipase are largely negative. A positive reaction to Bourne's test for ascorbic acid is given by many of the lipochondria.

SARCOMA 180 is one of the transplantable, non-metastasizing, connective tissue tumours of the mouse. A bit of sarcoma material, not exceeding 2 mm. in diameter, implanted beneath the skin of the breast as described by Zahl and Drasher (1947), grows within a fortnight into a conspicuous malignant tissue of such proportions that the host usually soon succumbs. Although studies have been made on the nuclear and cytoplasmic cytology of this and closely related tumours (Levine, 1939; MacCardle, 1951, and others), and Ludford and Smiles (1950) have recently compared the structure of mouse sarcoma cells with that of normal fibroblasts as seen in phase-contrast and ultra-violet micrographs, little pertinent work has been published regarding the finer cytological details and there is essentially no information available regarding the cytochemistry of the cytoplasm and its inclusions.

In the early part of the century there was a flurry of interest in the morphology of the cytoplasm of cancer cells, particularly with reference to the Golgi apparatus (see Cowdry, 1924, and Ludford, 1951, for many references). In these studies the Golgi network was frequently reported to be unusually elaborate in cancer cells in general. However, few modern cytologists support the 'network' theory of Golgi body structure and, considering the importance of the tumour cell in the economy of the animal body, a re-examination of the morphology of the cytoplasm of these cells, particularly with reference to the Golgi material, is desirable. Sarcoma 180 cells are particularly appropriate for study in this connexion because of the extreme malignancy of this tissue and the ease with which material of known age may be obtained and examined promptly or fixed for subsequent study.

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MATERIAL AND METHODS

All mice used in this study were males of the inbred Rockland Swiss strain, 18–22 grammes in weight, and were provided by the Haskins Laboratories, New York City. Mice were received within a few hours after the transfer of sarcoma tissue at the Haskins Laboratories. Slices of growing tumours were fixed, or studied without fixation, at varying intervals of from 1 to 12 days after implantation; but tumours 5 days after transfer have received the greatest amount of attention, for although these are generally not too large for optimum cytological fixation, a sufficient time after implantation has elapsed to ensure that the tumour is developing typically from the standpoint both of form and rate of growth. Furthermore, 5-day tumours have reached the steepest part of the weight-growth curve for this tumour (Zahl and Drasher, 1947). The 5-day tumour is usually roughly spherical or slightly ovoid and typically shows none of the irregularities in growth that are soon to develop.

The following methods were employed:

Vital staining. Bits of fresh tumour tissue were cultivated for from 2 to 5 hours at either 5° C. or 37° C., in either Tyrode's or Locke-Lewis's solution to which methylene blue, 1 part in 25,000, had been added. Other fragments were kept in Janus green B of similar concentration, and others for shorter intervals, up to one hour, in neutral red, 1 part in 50,000.

No appreciable difference was noted in the results obtained at the two different temperatures and in both cases many cells could be found that were believed to be viable because of the localization of the stain in discrete inclusions in the cytoplasm, without diffuse staining of either the cytoplasm or the nucleus. In the case of methylene blue and Janus green B, cells were interpreted as viable so long as the ground cytoplasm and nucleus continued to reduce the stain to the colourless form. Our observations on fresh material have been confined to those that could be made during the first 20 minutes after the preparation of a fresh mount.

Neutral red and methylene blue, used vitally, stain what we believe to be the same cytoplasmic elements to be described later, but while neutral red acts rapidly and methylene blue stains much more slowly, neutral red also rather quickly brings about an artificial enlargement of the inclusions to such a degree that such preparations are considered unreliable. Methylene blue, on the other hand, by behaving much more slowly, permits at least some observation to be made after the inclusions are stained but before any appreciable artificial enlargement occurs. There is no doubt, however, but that both of these vital dyes produce abnormalities when care is not taken to employ them at the minimum concentration necessary to stain the inclusions (see Dustin, 1947). It appears to us that much of the criticism of vital staining methods has been made by those who have been careless or indifferent in using them.

Our experience with Janus green B has been disappointing. Seldom have we been able to stain the mitochondria in cells which, through the failure of other elements to stain, could be considered viable. With the death of the

cell, however, as indicated by diffuse staining of both the cytoplasm and the nucleus, the mitochondria often appear deeply stained. Our experience with the methods we have employed is that the 'vital' staining of the mitochondria is typically a post-vital phenomenon in these cells. This, however, may be due to deficiencies in the brands of Janus green B we have used. Such preparations provide adequate data on the distribution within the cell of these inclusions, but considering the extreme lability of the mitochondria we are doubtful of the adequacy of such methods in determining mitochondrial form.

Unfixed and unstained tissue. Unstained living cells were also examined for comparison with the vitally stained and fixed tissue. These were studied both by ordinary microscopic methods and with phase-contrast equipment. In general, these methods have served largely to verify observations made by other means.

Permanent and semi-permanent preparations

For the demonstration of lipides, tissues were fixed in 10 per cent. formalin and in Baker's (1946) formaldehyde-calcium. For total fat content, frozen sections of tissue embedded in gelatine were stained with sudan IV, sudan black B in 70 per cent. alcohol, and in nile blue (sulphate) in distilled water. Sections were left in sudan IV overnight, were then washed in 70 per cent. alcohol and mounted in Farrants's medium. Sudan black B acted much more quickly, staining the lipides in 10 minutes or less. Sections were differentiated in 70 per cent. alcohol for a few minutes and then mounted in Farrants's medium for observation. A 1 per cent. solution of nile blue stained other sections in 5 minutes. These were then differentiated for 30 seconds to 1 minute in a 1 per cent. aqueous solution of glacial acetic acid and were then mounted for study in Farrants's medium.

Ice-cold acetone was employed as a fixative in a search for acid phosphatase, according to the method of Gomori (1941), as modified by Wolf, Kabat, and Newman (1943), and for the detection of lipase (esterase), following the method of Gomori (1945) and that of Wachstein (1946). Ice-cold acetone was also used as a fixative in an attempt to demonstrate sites of alkaline phosphatase activity by the azo-dye method of Seligman and Manheimer (1948). Acid phosphatase and esterase activity were also sought for by the azo-dye technique of Seligman and Manheimer (1949) and Seligman and Nachlas (1949). Rat duodenum, known for its high alkaline phosphatase activity, and rat liver, a tissue with high acid phosphatase and esterase activity, were used as controls.

Eighty per cent. alcohol was used as a fixative in the demonstration of organic iron, after the method of Mallory (1938). After acid hydrolysis Turnbull blue and Prussian blue preparations were exposed to MacCallum's haematoxylin to intensify the reaction. These preparations were subsequently subjected to treatment with hot ether/absolute ethyl alcohol (equal parts) for 30–40 minutes to extract all haematoxylin not in combination with iron compounds.

Helly's fixative was followed by Mallory's eosin methylene blue technique, by Sonnenblick's toluidin blue method, by haematoxylin/eosin/azure II, by the Masson method, and by the Feulgen and Giemsa methods. These preparations were used for general histological orientation, the demonstration of cytoplasmic basiphilia, and for determining the distribution of aldehydes. Slides incubated for 3 hours at 60° C. in 0.005 per cent. ribo-nuclease with a phosphate buffer were used as a check to determine whether or not the cytoplasmic basiphilia could be attributed to the ribonucleic acid content. Sections treated in this manner lacked the cytoplasmic basiphilia when stained subsequently with toluidin blue, eosin/methylene blue, and Giemsa. The basiphilia was retained in parallel control slides treated simultaneously in exactly the same manner except for the omission of ribonuclease from the hot buffer.

Flemming's solution (containing only 2 drops of glacial acetic acid per 5 ml. of fluid), followed by a 5-day treatment at 37° C. in 2 per cent. osmium tetroxide was used for the demonstration in permanent form of the Golgi material. It has been our experience that this technique generally results in little or no distortion of the Golgi elements as seen in fresh material. Their form in such preparations is also essentially identical to that found by vital staining with methylene blue. This procedure is, therefore, considered superior to the methods of Mann-Kopsch, Kolatchev, and Da Fano.

Tissue similarly fixed, but not post-osmicated, was followed by iron haematoxylin staining for the general relationship of cellular inclusions and nuclear structure. Similarly fixed tissue, post-chromed at 37° C. for 4-5 days in 3 per cent. potassium dichromate, was stained with Bensley's aniline acid fuchsin and methyl green in making permanent preparations of mitochondria.

Regaud's fluid, followed by an 8-day post-chroming in 3 per cent. potassium dichromate, was also used for the study of mitochondria after staining with aniline acid fuchsin and methyl green.

Rossman's fixing fluid, followed by the Feulgen-Bauer staining method (Bensley and Bensley, 1938) was used for the demonstration of glycogen.

The technique of Bourne (1936) was used on fresh frozen tissue in the demonstration of what we presume to be ascorbic acid.

OBSERVATIONS

Sections of 5-7 day tumours present a rather bewildering array of cells showing great variation in size and general structure. Zahl and Drasher (1947), whose terminology is followed here and whose description should be consulted for general structure of sarcoma 180, recognize at least two types of viable and presumably malignant cells which can be identified in fragmented, unstained fresh tissue. This is, however, a conservative classification which under-emphasizes the great variability particularly with regard to the cytoplasmic content as seen in suitably fixed preparations. There is, for example, a marked difference in total fat content in cells from different

regions. This component increases greatly as one passes in observation through the malignant cortex and approaches the boundary between this area and the necrotic centre. Cells of the intermediate zone between these two major areas have accumulated fat to a very marked degree. In sudan IV and Nile blue preparations, such cells comprise a conspicuous band immediately surrounding the caseous central mass (fig. 1, A and B).

Nevertheless, when the peripheral areas of the tumour at points of reaction with host tissues, the intermediate zone, and the caseous centre consisting largely of dead and dying cells (class B cells of Zahl and Drasher, 1947) are disregarded and attention is confined to the firm rind, cortex or peripheral zone, a degree of uniformity can be noted and the problem is simplified materially. Since it is this peripheral region that is considered to possess the greatest degree of malignancy and is therefore selected as the source of fragments for transfer, cells of this area may be considered more typical representatives of the malignant process from the standpoint of structure, tumour growth, and multiplication of cells. This is the region that has taken most of our attention, but it should be noted that a study which embraced all of the zones of the tumour from extreme periphery to caseous centre might prove exceedingly interesting since there is some indication that the position of the cell within the malignant mass is a rough index of its functional age.

Cytoplasmic basiphilia. Since the cytoplasmic basiphilia in these sarcoma cells can be abolished by treatment with ribonuclease, the basiphilia is presumed to be due to the presence of pentose nucleic acid. One of the most characteristic features of these cells is the pronounced basiphilia of the cytoplasm. This is usually diffuse and resembles the chromophilic substance which is found in the basal portion of normal pancreas acinar cells, although cells have been observed where the material appears to be arranged in the form of club-like rods, possibly to be identified as mitochondria. The basiphilic material appears to be more heavily concentrated at the periphery of the cell and towards the ends of cells which are unusually elongate.

Some, especially small, cells show the entire cytoplasm intensely basiphil, but in cells considered more typical the basiphilia is either much less intense or lacking altogether in a spherical area of variable size near the nucleus. This is the area where lipid is concentrated. Cells stained for basiphil cytoplasmic substances do not show the lipid as such, but its location is characteristically indicated by this very pale spherical area lying in the otherwise strongly basiphil cytoplasm (fig. 1, C and D).

These statements apply to the cells of the peripheral zone. As one passes inward toward the more necrotic central area, the cells lose their basiphilia rather abruptly and in the cells of the caseous centre, the cytoplasm and frequently the nucleus, are quite uniformly acidophilic. Pycnotic nuclei and formless nuclear materials remain basiphil, extracellular nuclear remains are strongly basiphil, but intracellular, possibly degenerating, nuclei often give a basic reaction. Cells in mitosis may show either strongly or weakly basiphil cytoplasm. Cells with acidophil nuclei have never been observed

undergoing mitosis. Amitotic division in cells of the caseous centre, however, is sometimes noted (fig. 2, A).

Golgi complex. Another striking feature of the principal cells of the sarcoma cortex is the elaborate character of the Golgi complex. It consists of homogeneous lipoid granules, recalling the Golgi 'presubstance' of Hirsch (1939), and larger bipartite spheres with osmiophilic surfaces and osmiophobic centres, recalling the Golgi 'systems' described by Hirsch (1939) for many kinds of animal cells. In life, these granules and droplets are pale yellow; they blacken with prolonged osmication (fig. 2, B and C); they stain very dark blue-grey with Baker's (1946) acid haematein test for phospholipides; they are generally dark blue with Nile blue; they are readily blackened with Sudan black B and they are stainable vitally both with neutral red and with methylene blue (fig. 2, D). These are typical examples of what Baker (1950) has called lipochondria. The striking similarity in the form and distribution of these Golgi bodies between suitably prepared osmicated cells and living cells vitally stained with methylene blue is illustrated in fig. 2, C and D (A, A₁; B, B₁; C, C₁). When the same picture is presented by fixed preparations and vitally stained living cells, one is justified in having a degree of confidence in the methods employed.

It is characteristic for these Golgi elements to occupy a roughly spherical area at one side of, and frequently partly within, a depression or hilus in the nucleus (fig. 3, A). This large area appears to consist largely of diffuse lipoid, probably composed of lipoid particles of submicroscopic dimensions. Baker's acid haematein test colours this area a diffuse grey-blue (fig. 3, B) and the whole spherical area with the discrete granules of lipoid suspended within is very reminiscent of Baker's (1944) interpretation of the 'Golgi element', in which both diffuse and concentrated lipoid was recognized. In some sarcoma cells, especially those that are unusually large, the lipoid zone occupies almost all of the cytoplasm (figs. 1, D and 3, C). The relative firmness of this lipoid mass is believed to be responsible for the frequently observed apparent distortion of the nucleus which involves the formation of a hilus (fig. 1, D).

No discrete lipoid zone seems to be identifiable in cells undergoing division. In such cells, individual Golgi bodies or spheres are widely scattered through

FIG. 1

A. Low power ($\times 100$) view of a frozen section through the centre of sarcoma 180 showing high total lipide concentration in cells of intermediate zone (A) immediately surrounding caseous, necrotic portion (B). The cells on each side of this marginal zone are relatively free of neutral fats. Nile blue.

B. High power ($\times 2000$) view of lipide-laden intermediate-zone cells closely surrounding caseous centre. Flemming's fluid followed by 5-day treatment with 2 per cent. osmium tetroxide.

C. Cytoplasmic basiphilia of cells of malignant cortex or peripheral zone. Note the peripheral distribution in each cell of the basiphilia and its absence from a large spherical area within the cytoplasm. Zenker fixation; Giemsa staining. Preparation of T. Sardinsky ($\times 1200$).

D. Same ($\times 2000$) showing similar relationship of parts in a giant tumour cell with unusually large pale lipide zone and peripheral dark-staining ribonucleic acid containing cytoplasm. Zenker fixation; Giemsa staining. Preparation of T. Sardinsky.

the cytoplasm and are not concentrated in a definite area. Both homogeneous granules and larger more complex forms are found, but the general impression is that cells undergoing mitosis contain fewer and smaller Golgi bodies. This is believed to account for the difficulty some investigators have experienced (Dalton, 1951) in demonstrating a Golgi 'network' during division. Small homogeneous Golgi bodies are not so readily distorted as are the larger forms. However, some Golgi material has been present in all of the cells of the cortex we have examined. During mitosis, the cytoplasm and cytoplasmic inclusions give the impression of being relatively inactive and there is no evidence whatever, from our observations, that the Golgi bodies divide during cell division.

With regard to the character and number of these lipid granules and spheres, two general areas of the malignant peripheral zone of the tumour can be recognized. In the more external areas, in which the cells themselves are smaller, the number and size of the Golgi bodies in each cell are proportionally less than in the deeper zone of somewhat larger cells. Likewise, in the deeper cells, the number of vesicular Golgi bodies (Golgi 'systems' of Hirsch, 1939) is correspondingly increased. Sudan black affects only the surface of these vesicular elements, leaving their centres unstained. With their increased numbers they occupy a greater mass of the cytoplasm.

The Golgi picture presented here deviates from the classical view of this inclusion as an 'apparatus' or 'network', but it is in general accord with the more modern accounts of its structure as presented by Hirsch (1939), Baker (1944), Thomas (1947), and others, contrary to the opinion of Dalton (1951) and MacCardle (1951). Our studies fail to reveal anything resembling a network in these sarcoma cells when they are examined either in the fresh condition or in most fixed preparations. However, such a network can be produced artificially in several ways. When the lipid inclusions are vitally stained with methylene blue, or post-vitally stained with Nile blue, or with Sudan black B, and the cell is subsequently shrunken by treatment with a hypertonic solution, the network makes its appearance and is the same colour as the colour of the stain employed. Moreover, this transformation can be followed while the Golgi material is under direct observation, proving clearly to the observer that the result is due to the shrinkage treatment (fig. 3, D).

FIG. 2

A. Cell of caseous centre (arrow) undergoing amitotic division ($\times 1200$). Baker's acid haematein method.

B. Cells of malignant cortex ($\times 1200$) showing characteristic distribution of Golgi material in spherical zone near nucleus. Flemming's fixation, followed by 5-day treatment with osmium tetroxide.

C. Same as B ($\times 1800$), especially for comparison with vitally stained cells of D. Note the close correspondence in the distribution of the osmiophilic (chromophilic) substances (A-A₁; B-B₁; C-C₁).

D. Cells vitally stained with methylene blue ($\times 1200$) for comparison with osmicated material of C. Note the close correspondence in distribution and appearance between vitally stained inclusions and osmiophilic substances of C (A-A₁; B-B₁; C-C₁).

Here, too, the Golgi 'network' is an artifact and it appears to us that conclusions drawn concerning the Golgi material, when this structure is interpreted as a 'network' such as those of MacCardle (1951), have little value.

Mitochondria. There is considerable variability in the mitochondrial picture in various cells of the tumour, just as there is in the other cytoplasmic components. Generally speaking, the mitochondria in fixed preparations appear spherical or rod-shaped. In cells post-vitally stained with Janus green B they are chiefly in the form of rods. Filamentous mitochondria have been observed only in cells in hypertonic media and we doubt if such forms occur in the living sarcoma cells. In what are considered to be the more typical cells, the mitochondria occupy a peripheral position, avoiding the more central Golgi zone. They form an incomplete halo around the lipoid zone with an interruption on the side of the lipoid area facing the nucleus (fig. 4, A). This region of the cell, as pointed out above, contains the heavy concentration of ribonucleic acid, as shown by its strongly basiphil character. In most cells, stained with acid fuchsin, a pale spherical area lying near the nucleus, apparently completely devoid of mitochondria but surrounded by them, is seen. In such cells a very clear and positive differentiation between the Golgi bodies and the mitochondria is readily apparent. Viewing such cells, one cannot help but think of the spherical Golgi bodies as representing aggregations of lipoidal substance in areas of diffuse lipoid, and of the mitochondria as somewhat similar aggregations of material within the ribonucleic acid containing cytoplasm (fig. 4, A).

Thus far things are fairly clear, but the relationship becomes less well defined when other tumour cells are examined. In some cells, particularly those of the intermediate zone between cortex and centre, Golgi bodies occupy most of the cytoplasm, while the volume of cytoplasm containing ribonucleic acid is greatly reduced. In these cells, the typical mitochondria are correspondingly reduced in number, but curiously many of what in other preparations are clearly to be classified as Golgi bodies are now fuchsinophilic. Golgi bodies are not fuchsinophil in the principal cells of the tumour, but, as is well known, they sometimes show this tinctorial property in other tissues. What interpretation is to be placed on this observation is not clear, but since the number of typical mitochondria in these cells is greatly reduced and because

FIG. 3

A. Cells of malignant cortex ($\times 1200$) showing relation frequently observed between nucleolus and extranuclear Golgi zone (arrows). In those cases where the nucleolus lies close to the nuclear membrane, the lipide zone almost invariably is found adjacent to the nucleolus. Flemming's fixation; Heidenhain's iron haematoxylin staining.

B. Cells of the malignant cortex ($\times 2000$) showing Golgi zone as seen in frozen section stained with Baker's acid haematein method. The nucleolus also gives a strong reaction.

C. Giant cell, similar to that of fig. D. In this case the Golgi zone is blackened with osmium tetroxide, which leaves the peripheral region of the cell unstained ($\times 1200$).

D. Golgi apparatus as seen in vitally stained material subsequently treated with hypertonic salt solution. The figures are typical of the classical conception of the Golgi apparatus, but the configuration is entirely artificial ($\times 1200$).

of the fuchsinophilic character of the Golgi bodies, it appears possible that as the lipoidal Golgi elements have extended out into the peripheral cytoplasm, some of the mitochondria have been adsorbed on their surfaces. Other possibilities are considered in the discussion.

Ascorbic acid. Bourne's (1936) technique for ascorbic acid is regarded as valid by some investigators, but has been criticized by others. We are not in a position to assess the value of this method as a histochemical test for this substance. However, a few years ago, one of us (Worley and Harary, 1946) noted a marked increase in the amount of argentophilic substances in the adrenal glands of animals subjected to treatment with large dosages of ascorbic acid. This is circumstantial evidence in support of the validity of the method.

Although Sluiter (1948) has been unable to confirm for the acinar cells of the pancreas the localization of ascorbic acid in the Golgi apparatus, contrary to the earlier findings of Hirsch (1939), Bourne (1936), Tonutti (1938), and others, there is a clear-cut positive reaction of the granules of the lipid zone of these tumour cells to the ascorbic acid test (fig. 4, B). Superficially, the test appears to be almost as satisfactory for the demonstration of the phospholipid spheres in the tumour cells as are the methods used for the demonstration of lipides. However, the lipide spheres demonstrated by the acidified silver method are less numerous, suggesting possibly different chemical properties for the more peripheral lipide inclusions. These inclusions also appear smaller with the ascorbic acid technique.

Lipase (esterase) and acid phosphatase. Our tests for lipase (esterase) and for acid phosphatase have been entirely negative for the cells of sarcoma 180. Tests for these substances were controlled by running parallel tests on tissues known to contain these materials. Positive results were obtained for the latter. Subsequently, Professor William Montagna, of Brown University, at our request, examined the sarcoma from this standpoint and he confirmed our findings. The junior author has just completed an extensive search for these substances in Professor Alex Novikoff's laboratory at the University of Vermont, using the azo-dye method of Seligman and Manheimer (1949), but again the results have been completely negative. We are forced to conclude that lipase (esterase) and acid phosphatase are not present in the cells of sarcoma 180 in quantities detectable by the cytochemical methods at present available.

FIG. 4

A. Large cell of malignant cortex of sarcoma 180 showing distribution of mitochondria in halo around the spherical Golgi zone ($\times 1200$). The relatively small size of the Golgi zone is believed to be due to the fact that the more peripheral Golgi spheres are fuchsinophilic (see text). Regaud fixation; Altmann aniline acid fuchsin staining. Preparation of N. Eisenberg.

B. Frozen section showing reaction of lipide zone to Bourne's technique for ascorbic acid ($\times 600$). The technique blackens the Golgi bodies.

C. Caseous centre ($\times 900$) showing extracellular Feulgen-positive substances released from necrotic cells. Zenker fixation; Feulgen technique. Compare with D.

D. Caseous centre ($\times 900$) as seen in vitally stained material showing extracellular droplets (arrows) believed to be the same as the Feulgen-positive material shown in C.

Glycogen. Whereas the majority of cells were entirely negative to the Feulgen-Bauer test for glycogen, an occasional cell gave a strong positive reaction, while control sections treated with saliva were always entirely negative. We conclude, therefore, that glycogen is of only sporadic occurrence in these cells and is not of regular occurrence in appreciable quantities.

Iron and alkaline phosphatase. The nuclei of sarcoma 180 cells, as is to be expected, give a moderate reaction to cytochemical tests for iron. A very strong positive reaction for alkaline phosphatase is shown by the walls of capillaries and small blood-vessels beyond the limits of the tumour, but not by those within, and the sarcoma cells themselves are entirely negative. Earlier studies with the method of Gomori have been supplemented by the azo-dye method of Seligman and Manheimer (1948), but without any positive reaction. Alkaline phosphatase, like acid phosphatase, is apparently not present in readily detectable quantities.

Desoxyribonucleic acid. All nuclei of the tumour give a strong positive reaction to the Feulgen test for aldehydes, as is to be anticipated. An interesting feature of the caseous centre of the tumour, however, is the very large quantity of Feulgen-positive material that is both extranuclear and extracellular, and which can be demonstrated in larger and larger quantities with the increasing age of the transplant. Thus, in the 12-day tumour, it is present in fixed preparations as an extensive network between cells (fig. 4, c), while it can also be noted in appreciable quantities in fresh preparations of necrotic tissue as spherical droplets (fig. 4, d). When such preparations are stained with such lipid-positive stains as sudan black B, these droplets remain colourless. The possibility that such discharged nuclear substances containing nucleic acids and nucleoproteins are of significance in the further growth of the tumour and the incorporation of host cells should not be overlooked, but in the present state of our knowledge of tumour growth it seems best to regard this phenomenon merely as the natural result of the disintegration of the nuclei of necrotic cells and of no further significance.

DISCUSSION

These sarcoma cells are exceedingly interesting because here we deal with a situation where abnormality is the rule and where, consequently, deviations from the normal are to be expected. Studies of such material conceivably can give us an insight into vital processes possibly not so evident in the normal tissue. Since, however, the abnormalities evidenced in the structure of the cells may be in any of several directions, the formulation of definite rules pertaining to the cytology of these cells is practically impossible. Levine (1939) has observed an almost endless variation in the nuclear pattern that such cells present, and we have encountered a somewhat similar situation for the cytoplasm.

A few fairly obvious conclusions can be drawn. These cells have an amazingly high lipid content which is indicated both by cells prepared for study of the total lipide and those prepared for study of the Golgi material.

Among the cells of vertebrates with which we are familiar, only oocytes and gland cells show such a concentration and degree of elaborateness as is demonstrated by the Golgi complex in these cells. Indeed, we have been repeatedly impressed with the general morphological resemblance between sarcoma cells and such gland cells as, for example, those of normal pancreatic acini.

We have also been impressed by the high ribonucleic acid content of the cytoplasm of these cells, again recalling the situation in vertebrate seromucous cells. Probably both of these conditions merely reflect the intense metabolism that is taking place and we have no direct evidence that any secretory product is being discharged. That certain storage products are being produced, however, is evident from the observation of the increased accumulation of lipides in cells as one approaches the caseous centre of the tumour.

In a general way, it is possible to distinguish three zones in the malignant portion of the tumour outside the caseous centre. (1) A more superficial zone, in which the cells and their nuclei are smaller on the average and in which the Golgi bodies or lipochondria are principally in the form of small, homogeneous granules. These cells closely resemble normal fibroblasts. (2) A broad, more central zone of larger cells, somewhat resembling normal fibroblasts but much larger, and in which many of the Golgi bodies display chromophobic interiors. (3) An intermediate zone, adjacent to the necrotic centre, in which the Golgi bodies are fewer in number but in which neutral fats and lipides are abundant. We suggest that the cells of zone 1 are to be interpreted as relatively young cells while those of zone 3 are much older cells at a stage just before they become necrotic, become incorporated into the caseous centre, and eventually die or disintegrate. The tumour as a whole, in the meantime, is expanding farther and farther into the host tissue.

Our observations support Hirsch's (1939) view that the Golgi bodies undergo transformation and can be classified into different types which are chronologically related. Clearly they show variation in size which can be noted in almost any type of preparation. Now, when the same technique is applied to these elements of different sizes, the smaller ones appear homogeneous, but the larger ones exhibit pale 'chromophobic' or 'osmiophobic' centres. It is believed that when these larger elements are 'fixed' or otherwise disturbed, the chromophilic or osmiophilic envelope surrounding the chromophobic core is artificially contracted or slipped off from the latter, thus rendering it visible. Such a body, consisting of a chromophobic sphere, capped or partly surrounded by a chromophilic knob, crescent, or ring, is believed to be an artifact; but this should not be taken to mean that the chromophobic core in contact with the chromophilic surface does not exist, as some investigators have either implied or suggested.

Holtfreter (1946) suggested that bipartite Golgi bodies of this sort represented 'desolvated' lipid spheres. Others have referred to such bodies as 'invaginated' mitochondria. Neither interpretation demands that 'desolvation'

or 'cavulation' is always to be considered an artificial process, although it has been shown that such appearances can be produced artificially by certain appropriate external agents. In view of the fact that 'desolvation' seems only to involve the larger, and perhaps more 'mature' lipid spheres, and considering that in the eggs of molluscs (Worley and Worley, 1943) and elsewhere these chromophobic cores eventually become free of their chromophilic envelopes during the course of development or activity, for the time being it would seem pertinent to regard 'cavulation' or 'desolvation' as a process that may occur naturally in the cell from time to time as the natural metabolic processes of the cell warrant and permit. 'Cavulation' occurs so readily in the larger Golgi bodies whenever the cell is disturbed in any manner that it seems unlikely that it does not occur naturally from time to time in the normal healthy cell under certain given conditions. Xeros (1951) has recently confirmed that chromophobic centres of this sort are found in some of the Golgi bodies of the pancreas. Many investigators (Hirsch, 1939; Thomas, 1947; Siang-Hsu, 1947; Bourne, 1951, and others) have regarded this as the natural manner in which the secretory granules and certain storage products arise. In the present state of our knowledge of lipid metabolism, however, it would be premature to offer comment regarding the nature of the chemical processes involved.

Some of the observations recorded here suggest that Golgi bodies and mitochondria may at times form complexes between themselves or that mitochondrial material may become incorporated into the Golgi spheres. Certain workers in the past, notably Parat (1928) and more recently Adamstone (1950) have made similar suggestions. Oliver (1948) has furnished a most convincing demonstration of this in cells of the mammalian kidney where the neutral red staining inclusions apparently incorporate mitochondrial stuffs. This is not the usual interpretation, but in these cancerous tissues we are dealing with abnormal cells where peculiar happenings should not be unexpected. The fact that such an occurrence may take place in abnormal cells suggests the possibility that normal cells may occasionally behave in a similar manner, at least in particular instances. In this connexion, it is worth while to recall the conditions in the mammalian mast cell. As is well known, such cells are loaded with mast granules. Techniques for the Golgi material demonstrate only the mast granules while when techniques for mitochondria are employed, again it is the mast granules that react (Montagna and Noback, 1948). Here is a case where it appears that one type of inclusion is structurally, and probably functionally, endowed with the attributes that in most cells are associated with two distinct types of inclusions. It would not seem appropriate to assume *a priori*, that in all animal cells different functions are invariably carried out by structurally unrelated cytoplasmic organelles.

The observation that in these tumour cells the nucleolus is often seen pressed against the nuclear membrane adjacent to the extranuclear lipid mass calls for brief comment. This seems to be not an uncommon situation in cells where lipid metabolism is proceeding at an unusually high rate. Such or a similar

situation has been repeatedly detected in developing oocytes (Gardiner, 1927; Saguchi, 1927, 1928; Hirschler, 1929; Singh and Boyle, 1938; Fahmy, 1949); these are cells in which intense lipoid metabolism is associated with the accumulation of cytoplasmic 'fatty' yolk. Although the nucleolus itself is not generally considered to contain much if any lipoid, this intimate association occurs far too frequently to be considered purely coincidental.

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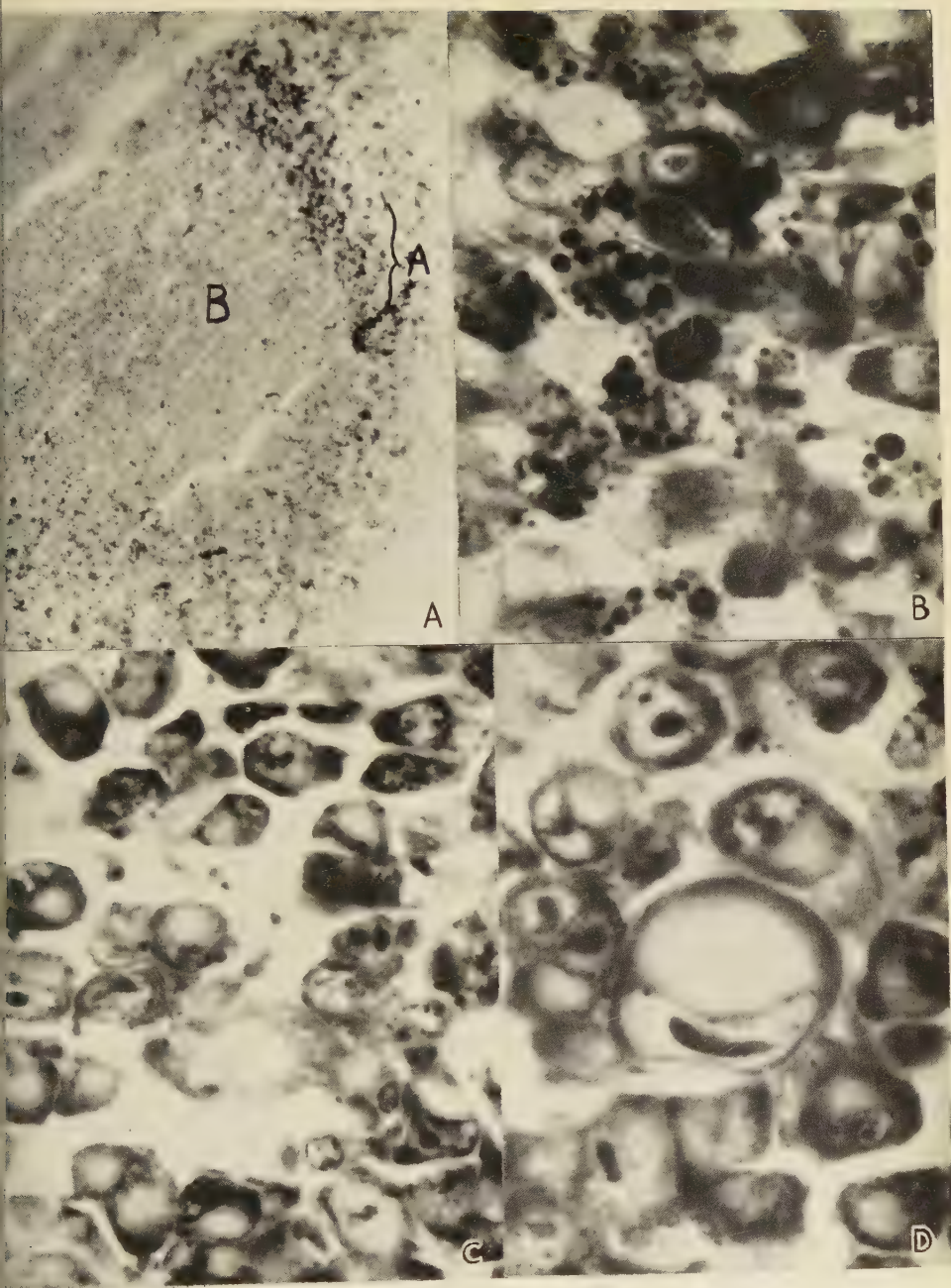


FIG. 1

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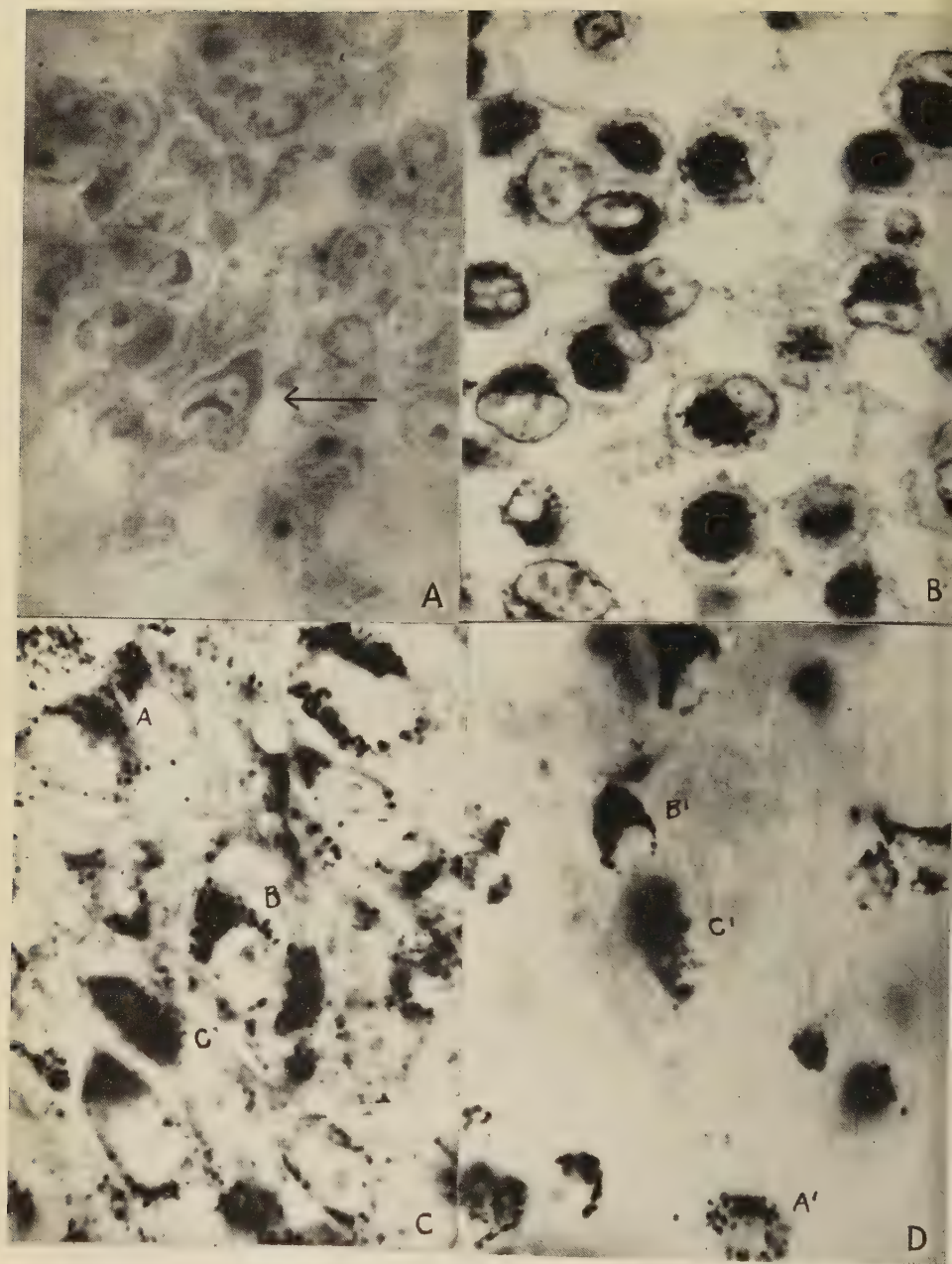


FIG. 2

L. G. WORLEY and H. W. SPATER

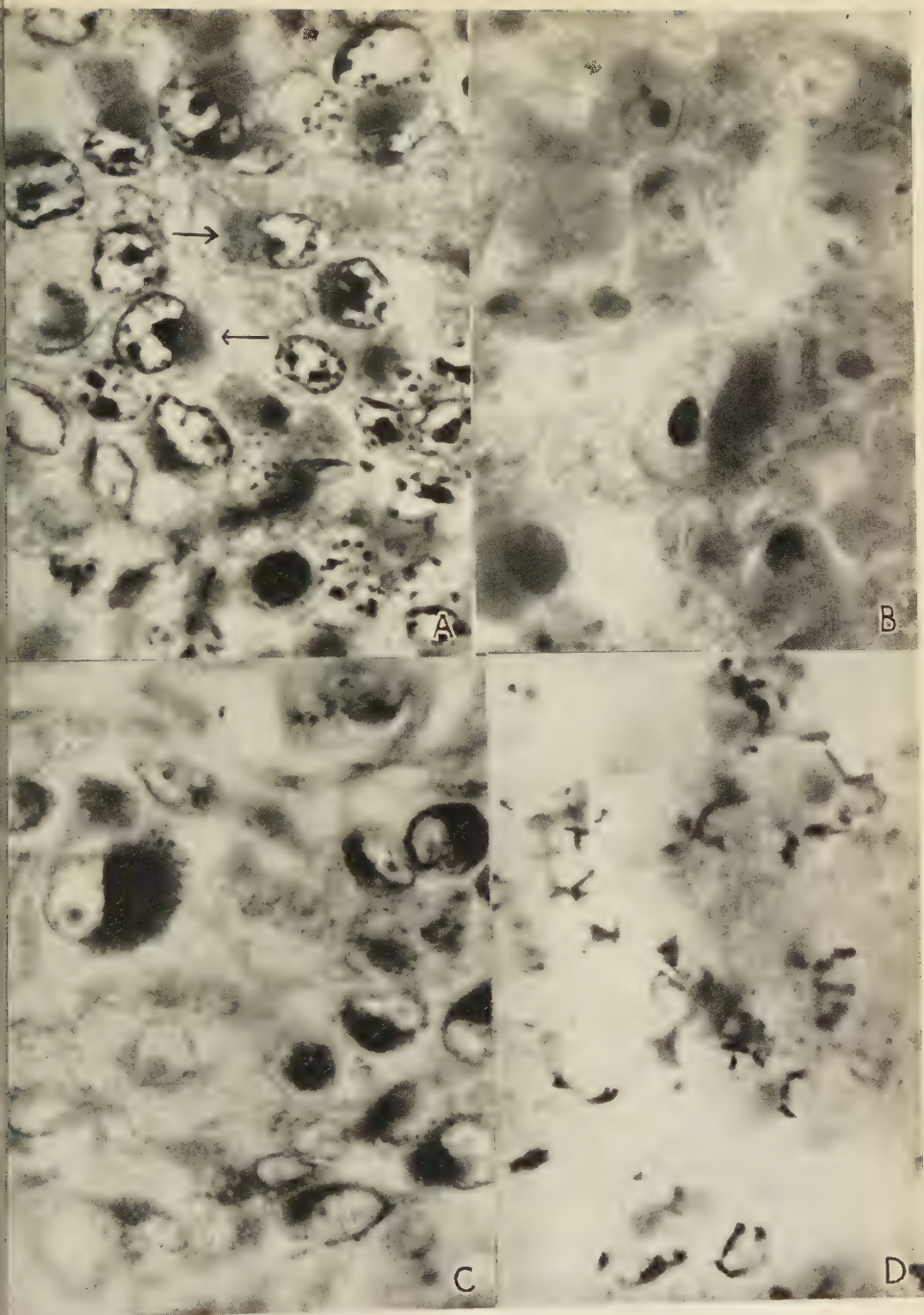


FIG. 3

L. G. WORLEY and H. W. SPATER

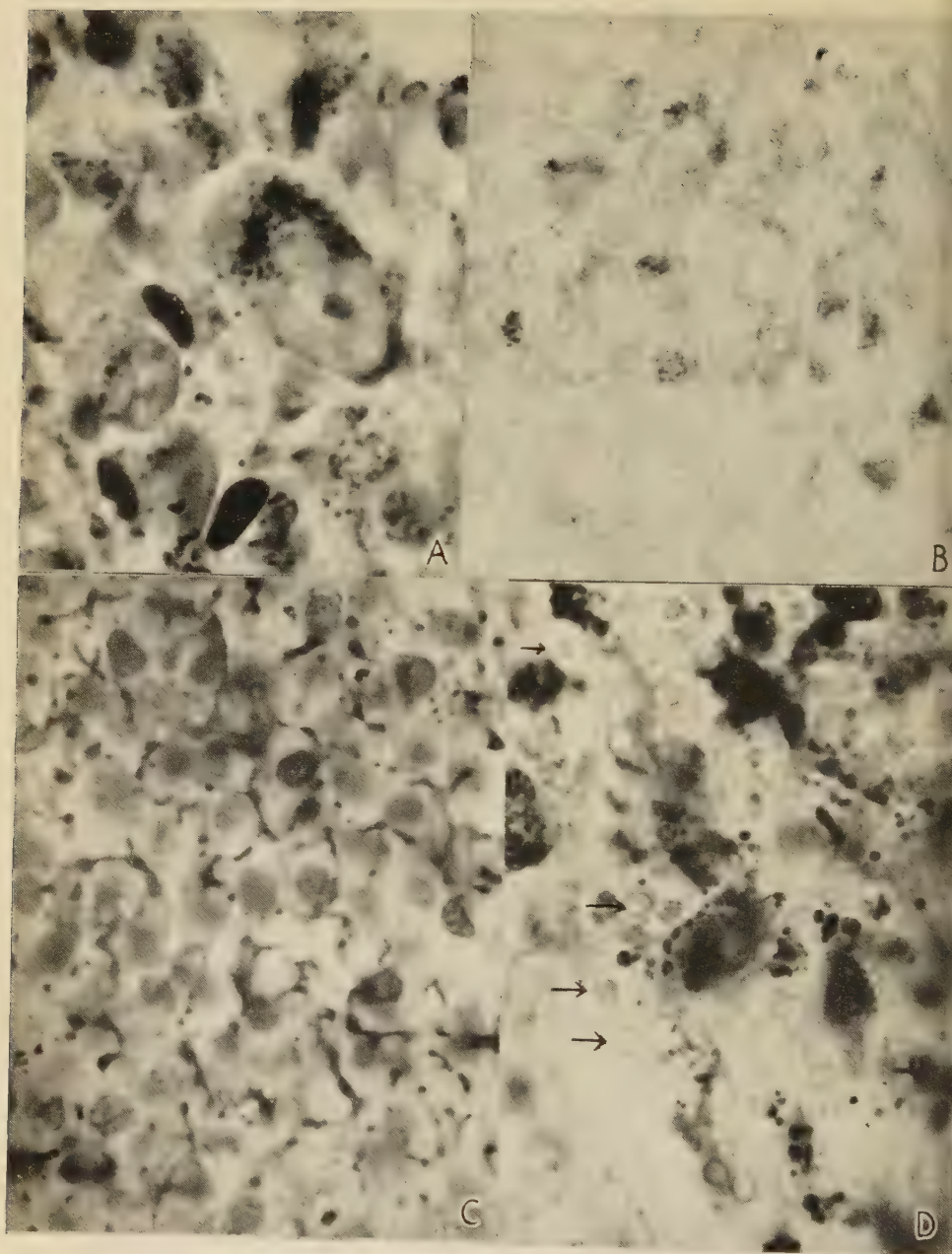


FIG. 4

L. G. WORLEY and H. W. SPATER

Amoeba hugonis n.sp.: Life-history

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SUMMARY

Material collected in Loch Tannoch was allowed to macerate in a chemical nutrient. A rich crop of *Euglena gracilis* as well as other infusoria resulted. Eight months later, when the *Euglena* had encysted, many amoebae were found at the bottom of the receptacle. They constitute a new species, here named *Amoeba hugonis*. An average adult specimen, when extended, measures about $104 \times 5.2 \mu$. The nucleus consists of a central karyosome lying in the nuclear sap, separated from the cytoplasm by a well-marked nuclear membrane. Between the latter and the karyosome is situated an chromatic 'collar' with chromatin particles embedded in it.

Binary fission is described, but a study of mitosis has been deferred. The life-history of this small amoeba is very similar to that of the large *A. proteus*, &c. The cycle occupies two months. Chromidia begin to appear in the cytoplasm of the early adult. They give rise to spores, out of which amoebulae hatch.

INTRODUCTION

MATERIAL from Loch Tannoch, Milngavie, was collected in May 1947 in the hope that it would yield *Amoeba nobilis* and *A. villosa*, as a few specimens had turned up in previous catches and in both these species there remains much to be worked out of their respective life-histories. The catch was put into a trough containing the following inorganic nutrient:

magnesium sulphate	.	.	5 gm.
'double superphosphate'	.	.	7 gm.
potassium nitrate	.	.	17 gm.
ammonium sulphate	.	.	5 gm.
water	.	.	18 litres

Organic food was not provided, as the maceration of the vegetable matter present would provide enough of this for any micro-organisms. Five months later the culture was swarming with euglenoids. Later examination revealed neither of the two species of amoebae, but the culture was retained. A subsequent examination showed that many euglenoids were encysting and that many other micro-organisms were present. Although no *Amoeba nobilis* or *A. villosa* could be found, yet the material was retained and entered in our field book as culture 118. On 24 January 1948 there was a very large crop of small amoeba (easily visible under a 'No. 3' objective). It was so reminiscent of *A. villosa* that one hoped it was the young of that species. The creatures were feeding heavily on golden brown spherical masses very similar in size and appearance to those figured by Leidy (1879, pls. I and II) and which have also

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been recorded as present in *A. villosa* and *A. nobilis* in our field book entries. Because these spherical masses are so favourite a diet, it seemed to be desirable to cultivate them. But the problem was, were they encysted infusoria, or algal spores (as suggested by Leidy)? Zoological and botanical friends were consulted, but no satisfactory explanation was forthcoming at the time and I did not succeed in cultivating the spheres. Much later on, however, golden coloured spherules began to appear in the *Euglena gracilis* of culture 118. I consulted Dr. Margaret Jepps who called my attention to a paper by Dr. Pringsheim 'Les relations de parente entre Astasiacees et Euglencees', 1950, in which he referred to *Euglena gracilis* var. *fuscopunctata*, which tended to form 'des corps brun'. I submitted to Dr. Pringsheim *E. gracilis* from my culture 118. He immediately made a culture of them, thus forming the 25th variety of *E. gracilis* acquired by him. He pronounced these structures to contain carotene; whether they were wholly carotene or a coating of carotene over an underlying substratum he did not know. This pigmented material is very similar to, if not identical with, that of the eye spot of euglena. Dr. Pringsheim suggested that the euglenae of culture 118 were not perfectly healthy, probably owing to deficient nutrition. I can corroborate this by my observation that when the *E. gracilis* are increasing rapidly they are quite free from these brownish bodies. I have also found similar bodies living on clotted masses of decaying vegetation.

To return to the amoebae—if these were the young of *A. villosa* they normally would be adult in 2 or 3 months. All amoebae are proverbially slow growers. The largest specimen of the amoeba was quite as large as the youngest stained specimen of *A. villosa* which we possessed. Unfortunately no living *A. villosa* could be found in the ponds for purposes of comparison. To anticipate what will be detailed later on, no adult *A. villosa* have appeared in culture 118 in the four years that it has been under constant observation. Assuming that this unknown amoeba was new to science, and has not been described before, I propose the name *Amoeba hugonis* to honour the Dean of the Graduate College, Princeton, Dr. Hugh S. Taylor.

In his account of *A. villosa* Leidy describes and figures (pl. VIII, figs. 1–16) several small amoebae which he suggests are the young of *A. villosa*. These figures bear a slight resemblance to *A. hugonis*, especially the one with the large nucleus surrounded by a halo (p. 64, fig. 10, pl. VIII). In the writings of early observers, Carter (1863), Wallich (1863), and Duncan (1877), figures similar to *A. hugonis* also appear. Fortunately another search in Loch Tannoch resulted in a catch of young *A. villosa*, so that I can definitely state that the amoebae figured by the above authors are neither *A. villosa* nor *A. hugonis*.

LIFE-HISTORY OF *AMOEBA HUGONIS*

To facilitate observation, material from stock was cultivated in Petri dishes $4 \times \frac{1}{4}$ in. and a small quantity of wheat was added to the pabulum of chemical nutrients. With a one-seventh in. water immersion objective the various stages of growth of the amoebae can readily be studied in the living animal in

sequence. These were supplemented by studies of the amoebae on slides with coverslips. Frequent use was also made of the aceto-carminic technique; i.e. amoebae were left overnight, or longer, in acetic alcohol (equal parts of strong acetic acid and absolute alcohol) and then stained in aceto-carminic. This is the most efficient method of detecting chromatin. Coverslip preparations made by fixation in Bouin's fluid and stained in Ehrlich's haematoxylin were also used.

The average adult *Amoeba hugonis* measures about $104 \times 5.2 \mu$ when extended; a thick, well-nourished individual, not creeping quickly, measures

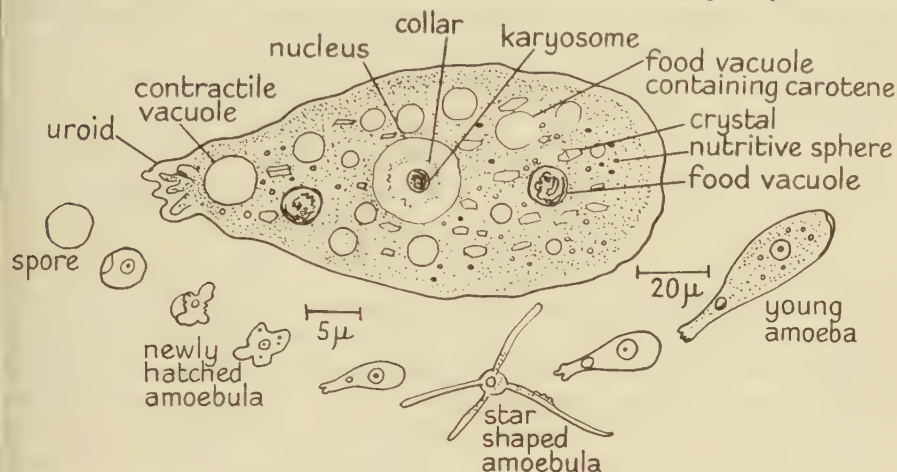


FIG. 1. *Amoeba hugonis*. Above, adult form. Below, early stages in development from spore.

about $84 \times 28 \mu$. This species is often limax-like when moving actively and has a distinct uroid; but blunt pseudopodia cause the amoeba to become more or less palmate. It often becomes sluggish and more or less spherical with a crenate margin and a conspicuous vacuole (cf. Leidy's fig. 8, pl. VIII). When it is in this condition any change in the surrounding water causes the vacuole to enlarge abnormally and eventually the creature dies. I have lost one or two Petri dish cultures put out for study by doing what can be done safely with most amoebae, adding Glasgow tap-water. Dilute chemical nutrient water must be used for replenishing the culture water, or replacing the water on the slide due to loss by evaporation.

The consistency of the cytoplasm seems to be greater than any we have investigated hitherto, and it has a pale bluish tint and is much more transparent and limpid than that of *A. proteus*, *A. discoides*, *A. leschaeae*, or *A. kerrii* (fig. 1). The cytoplasmic contents stand out clearly. The ectoplasm shows up clearly only when the creature is moving. The nucleus is large by contrast and is easily visible, consisting of a central karyosome lying in the nuclear sap, with a well-marked nuclear membrane. The 'collar', i.e. a thin layer of nuclear material situated at varying distances between the karyosome and the nuclear membrane, is best studied in stained preparations. The thin

disk-shaped nucleus is not rolled into 'plan' and 'elevation' positions by the moving endoplasm. The 'plan' view is always visible, in contrast to what obtains in the nuclei of the large *Amoeba proteus*, *kerrii*, &c.

Crystals of various shape are numerous and large by contrast with those of *A. proteus* and they give the cytoplasm a very coarse granular character in the adult *A. hugonis*. As the amoeba becomes more aged small nutritive spheres also appear. Growth is a matter of increase in bulk rather than in surface area. *A. hugonis* feeds greedily on the golden brown spherules already referred to and is a very striking creature when well fed with these and euglenoids. It has a marked capacity for finding these bodies, even when they are very sparse in the culture. But I have not been able to decide whether those engulfed by the amoeba are derived from the digested 'infected' euglena



FIG. 2. Fission of *A. hugonis*.

or taken freely. The small particles derived from the eye-spot of euglena would seem to be the slowest part of an ingested euglena to be assimilated by the amoeba. According to Dr. Pringsheim such red substances are very durable, being embedded or dissolved in a wax-like matrix similar to that causing the preservation of pollen grains in a fossil state. The length of time taken by the amoeba to digest and assimilate the contained carotene I have not been able to determine. *A. hugonis* ingests ciliates and flagellates, its appearance varying somewhat according to its food. The contractile vacuole is conspicuous and is to be found at the posterior end where it evacuates very slowly and deliberately, just in front of the uroid. Usually the amoebae do not move about very much, and they are distinctly gregarious, occurring in dense patches in some parts of the culture dish while other patches are quite clear of them. And this gregarious character is true of all the stages.

Fission (fig. 2).

This can be witnessed in the living animal with the one-seventh in. water immersion lens. When about to divide the amoeba becomes spherical with short protruding pseudopodia. From the centre of this mass a wide projection slowly emerges, down which protoplasm flows to form another spherical mass, still connected with the parent by a wide neck. This connexion is gradually severed and two amoebae result. Before separating, however, these two masses come together again before finally flowing away as two daughter amoebae. The whole process occupies about half an hour.

Stained preparation of adult

The fixatives employed were Bouin's fluid and acetic alcohol. It is principally to show nuclear structure that fixed specimens were needed. The

alcoholic technique particularly was useful for the life-cycle stages in demonstrating chromidia and chromatin blocks.

An adult nucleus of *A. hugonis* (fig. 1) measures 9.6μ in diameter, its karyosome being 3.2μ and the 'collar' 4.8μ . The staining capacity of the karyosome varies with the physiological state of the amoeba, as does its size, and the number of discrete blocks of chromatin. The structure named the 'collar' lies between the karyosome and the nuclear membrane. It consists of an achromatic ground work on which are to be found, especially in older amoebae, discrete particles of chromatin. When the stain has been Ehrlich's haematoxylin and light green, the vivid green of the achromatic parts of the nucleus makes a sharp contrast with the chromatin. The position of the 'collar' varies in different individuals: sometimes it is close to the karyosome, at other times it is nearer to the membrane, all intermediate positions between these two extremes being recorded.

Reproductive cycle

In Petri dishes which contained adult *A. hugonis* of uniform age it was found repeatedly that when they reached an average of about $104 \times 5\mu$, and when their cytoplasm was packed with crystals and minute nutritive spheres, they all disappeared. At first it was concluded that the cultures had died out owing to faulty technique; but when it was found that after a period of time varying from 2 months upwards the amoebae reappeared and flourished, this conclusion was seen to be erroneous, as well as the belief that they might be the young of *A. villosa*. It was decided that in their disappearance and reappearance and in the depression and optimum periods of the culture, one was witnessing the life-cycle of a new species.

It was of interest to have material to investigate, so much smaller in size than any of the free-living amoebae whose life-cycle had already been worked out. In *Amoeba proteus*, *discoidea*, *lescheriae*, and *kerrii* sections sometimes reveal the fact that occasionally portions of the nuclear membrane dissolve to allow chromidia to escape into the cytoplasm. In *A. hugonis*, stained preparations of senile individuals in which all the crystals had been dissolved revealed numerous chromidia. At the same time the nuclear membrane is unusually transparent, and the 'collar' well stocked with chromatin blocks very near to the membrane, and chromidia very densely massed in the neighbourhood, outside the thin nuclear membrane. In older specimens there were small cysts. Because the term *cyst* has been used for different structures in amoeba by different authors, I have followed Dr. Margaret Jepps in applying the term *spore* to the small reproductive bodies out of which amoebulae hatch. It is when *A. hugonis* are stocked with spores that they disappear from the culture, a period of depression ensuing, the cytoplasm disintegrating, the released spores falling to the bottom of the dish. I have not yet discovered whether or no the spores survive drying, as in the case of *A. proteus* (Galbraith and Taylor, 1950).

Excystation and development of the young A. hugonis (fig. 1.)

A scrutiny of the floor of a Petri dish culture in a 'depression' period reveals cysts of various organisms, those of moulds and some flagellates being very similar to the spores of *A. hugonis*. Excystation of the latter, which measure 5μ to 6μ , is heralded by the appearance in the spore of a small contractile vacuole, beating rhythmically, indicating that the spore wall is ruptured. The contractile vacuole becomes larger and gradually an oily looking young amoeba makes its way out of the spore and creeps slowly away on the matted mould hyphae and bacterial debris. These amoebae are difficult to study unless one rubs a clear patch on the bottom of the Petri dish on to which they can creep. The uroid is present and the nucleus can be distinguished. The cytoplasm, homogeneous at hatching, now becomes mottled with food vacuoles containing minute organisms. These amoebae now become star-shaped, especially in a strong light. Very long hair-like, blunt pseudopodia are formed, and all the amoebae float up to the surface. They then withdraw these pseudopodia and sink down to the substratum, creep and feed and grow, the cytoplasm being very mobile at this age. Amoebae which hatched out on 26 February were $28 \times 11.2\mu$ on 10 April. Now their cytoplasm begins to get more viscid and the ectoplasm seems invisible except at the tip of the pseudopods. They often round themselves off. They are very gregarious and grow quickly from now onwards, especially if the golden-brown spheres are available as food. By 28 April they were undergoing fission and the culture was near its optimum.

DIAGNOSIS

Found among thick masses of encysted *Euglena gracilis* (normal form) and the variety *E. gracilis fusca-punctata*. Size increases with age: a stained adult having average dimensions $98 \times 64\mu$; nucleus 8μ in diameter, consisting of nuclear membrane, a centrally placed karyosome (3.2μ), and a collar of achromatic material 4.8μ in diameter. The nucleus, a thin disk in shape, is easily visible; it is not rolled over by the flowing endoplasm into alternate 'plan' and 'elevation' position. Chromatin particles of various sizes in karyosome and 'collar'. Cytoplasm coarsely granular because of numerous crystals, small nutritive spheres. One contractile vacuole which when of maximum size is situated between the nucleus and uroid. A gregarious amoeba which feeds on the golden spherules and eye-spots of *E. euglena* (var. *fusca punctata*), euglenoids, and infusoria. Divides by fission. No sexual cycle, spores formed from chromidia, which produce amoebulae that become adult in two months.

This amoeba can readily be distinguished from *Amoeba dubia*, *lescherae*, *kerrii*, *proteus*, *discoides*, *villosa*, *nobilis*, and *Pelomyxa carolinensis* (*Chaos chaos* of Schaeffer), by its size. The amoebae enumerated above are just visible to the naked eye when adult, whereas this amoeba can be detected only with a 'No. 3' objective. Moreover, the nucleus of this amoeba differs from the adult nucleus of those enumerated above. *Amoeba nobilis* and *Pelomyxa carolinensis* are multinucleate, this amoeba is uninucleate. It is true

that the nucleus of this amoeba is similar to the developing nucleus of *A. lescherae*, *kerrii*, *discoides*, *proteus*, but this nucleus completes its life-cycle without metamorphosing into the form characteristic of the adult of the above enumerated four species. As pointed out in the text, there is a resemblance between this amoeba and what Leidy describes and figures as 'probably the young of *A. villosa*', but this amoeba never grows up into *A. villosa*.

The blunt pseudopodia indicate at once that this amoeba is not one of the commonly occurring amoebae of Schaeffer's genus *Mayorella*.

A careful perusal of Schaeffer's figures and descriptions of thirty-nine new marine and fresh-water species, and Penard's (1902) new species, none of which I have had an opportunity of examining alive, convinces me that this amoeba is not one of them. Therefore, I conclude that this amoeba is new to science.

I am very grateful to my friend Mr. Ronald Graham Kerr for preparing fig. 1.

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The Development and Structure of the Anterior Region of the Body in the Sabellariidae, with special reference to *Phragmatopoma californica*

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SUMMARY

Attention is drawn to the confusion which has been caused by the loose terminology of the anterior appendages in polychaetes, and more exact connotations are proposed. As the prostomium can be recognized as a comparable unit throughout the Polychaeta, a consideration of its constitution is deferred.

In the present paper the development and constitution of the anterior region of the sabellariid worms are considered. The larval development of *Phragmatopoma californica* (representing the most highly advanced genus) is described, and the structure of the adult of this species is compared with that of species belonging to other genera.

It is concluded that the opercular stalk arises mainly from the first segment; that the opercular paleae represent the notochaetae of the first two segments, and that the oral tentacles and the building organ are also developed from the first segment. The prostomium bears a single pair of tentacles.

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INTRODUCTION

THE body of all polychaetes consists of a series of segments bounded by non-segmental regions; the prostomium anteriorly, the pygidium posteriorly. Each segment bears a pair of lateral lobes or parapodia from each of which extend two sheaves of chaetae. In the anterior region, this plan is usually modified, the segments often being fused one with another and to the prostomium, so that the homologies of the different parts are obscured. Much confusion has arisen regarding the terms used to denote the anterior appendages, and it is the purpose of the present studies to clarify the meaning of such terms as 'palps', 'tentacles', and 'cirri', and to investigate the fate and structure of the most anterior segments.

The following nomenclature is proposed. The *prostomium* is that region of [Quarterly Journal of Microscopical Science, Vol. 93, part 4, pp. 435-452, Dec. 1952.]

the body which lies anterior to the mouth and contains the brain, bears the eyes (when present), and presents no obvious signs of a segmental nature. Typically, it bears a pair of *palps* ventrally, and a number of *antennae* dorsally. The palps are innervated by nerves arising usually from the ventral or ventro-frontal region of the brain, the antennae from the dorsal or dorso-frontal region of the brain. In doubtful cases, appendages which might be referred to as either palps or antennae should be called merely 'prostomial appendages'. Hypothetically, a *segment* is a unit containing the coelom arising from paired mesodermal pouches which open to the exterior by a pair of segmental organs or nephridia, provided with one or more pairs of nerves arising from a ganglion in the ventral nerve chain, blood-vessels and gonads, and which is provided with two sheaves of chaetae on each side. In all modern polychaetes this plan is greatly modified, especially in the anterior region where the presence of a segment may be indicated by the nervous system alone. It does not, of course, follow that all such nerves in the anterior region are segmental in origin. Each segment is provided with a single pair of lateral parapodia, each bearing two sheaves of chaetae; those arising dorsally being *notochaetae* and usually emerging from the dorsal lobe of the parapodium or *notopodium*; those arising ventrally termed *neurochaetae* usually emerge from the ventral lobe or *neuropodium*. Both noto- and neuropodia bear a single *cirrus*, that arising from the notopodium being called the notopodial or *dorsal cirrus*, that from the neuropodium, the neuropodial or *ventral cirrus*. The term 'peristomium' is a useful but somewhat ambiguous term, and when used should be clearly defined, since although in most instances it is synonymous with the first segment, in some polychaetes, such as nereids, what is commonly called the *peristomium* consists of two segments fused together. The term *tentacle* should be used only as a modifying word to the terms already proposed (thus: 'antennal tentacles'; 'tentacular cirrus', &c.), and to filamentous structures (such as the oral tentacles of sabellariids) which cannot be homologized with any of the structures as here defined. The segment bordering, surrounding, or apparently immediately behind the mouth will be referred to here as the first segment.

Most workers seems to agree that the polychaete brain consists of three parts (Racovitza, 1896; Goodrich, 1898; Hanström, 1928; Snodgrass, 1938; Raw, 1949); but opinions differ as to whether the whole brain is presegmental or whether segmental ganglia have been added on from behind. The eversible proboscis present in many polychaetes has also been assigned a segmental origin by the Stanford school (Henry, 1947 *a, b*), and by Raw (1949). Nevertheless, whatever the constitution of the prostomium may be, it is quite clearly a unit comparable throughout the group; so a discussion of this subject and the origin of the proboscis may be deferred to a later paper.

The Sabellariidae are one of the most specialized families of polychaetes. One of their many interesting features is the development of an operculum from the anterior segments which in the most advanced genera completely obscures the prostomium. The operculum bears two sheaves of large chaetae or paleae on each side, and though little developed in the most primitive

genera, in the more advanced members of the family they are greatly enlarged and arranged in a radial manner on the top of the opercular stalk to form a protective crown or stopper to the tube when the worm retracts. The constitution of this operculum and the homologies of the opercular paleae have attracted the attention of previous workers (Meyer, 1887, 1888; Gravier, 1909; Johansson, 1927; Hartman, 1944), but each has arrived at somewhat different conclusions. It is interesting, therefore, to see how this region develops. All sabellariids have a long development in the plankton, their larvae often being common in many parts of the world though frequently confused with those of spionids, which they superficially resemble. Caullery (1914) was the first to distinguish the larvae of the two families, whilst Wilson (1929) made the first detailed study of the larval development (*Sabellaria alveolata* and *S. spinulosa*). Hartman (1944) gives a short account of the development of *Phragmatopoma californica*, and previous references to the larval stages of sabellariids will be found in this and in Wilson's paper. The development of this latter species is here described in greater detail with especial reference to the elaboration of the anterior region, and this is followed by a description and discussion of the structure of the anterior region of sabellariids in general.

Fertilizations of *Phragmatopoma californica* were achieved by mixing the ripe eggs and sperm, and decanting into larger vessels the young larvae that swam to the surface. Further supplies of larvae were obtained in tow-nettings taken just offshore at La Jolla, California. The structure of the adults of this and other species was studied by dissection, thick sections and histological sections. All the drawings of larvae have been made from living specimens narcotized by 7 per cent. magnesium chloride, with the aid of a camera lucida or eye-piece graticule and squared paper. The drawings of adults were made from individuals fixed in Bouin's fluid after narcotization with magnesium chloride. No noticeable contraction of the tentacles took place on fixation, and the appearance of the anterior part of the body as shown in fig. 12 is closely similar to that of the living animal. The sections were drawn with the aid of a microprojector.

THE DEVELOPMENT AND STRUCTURE OF THE ANTERIOR REGION OF *PHRAGMATOPOMA CALIFORNICA* (FEWKES)

The larval development of *Phragmatopoma californica* follows the same pattern as that described by Wilson for *Sabellaria alveolata* and *S. spinulosa*.

Males and females occur in approximately equal numbers. The eggs when mature are purple in colour and about 75μ across. The young trochophores begin revolving after about 12 hours and after 24 hours a recognizable proto-troch has developed and the larvae can then swim off the bottom (fig. 1, A). At this time a central mass of megameres may be seen to be surrounded by a transparent layer of smaller cells containing groups of yellow pigment-granules, the number of these patches being somewhat variable. By the second

day the prototroch is better developed but is incomplete mid-dorsally and remains so throughout life. The gut which has by now appeared soon acquires a stomodeal opening, and a number of chaetae are erupted from the postero-

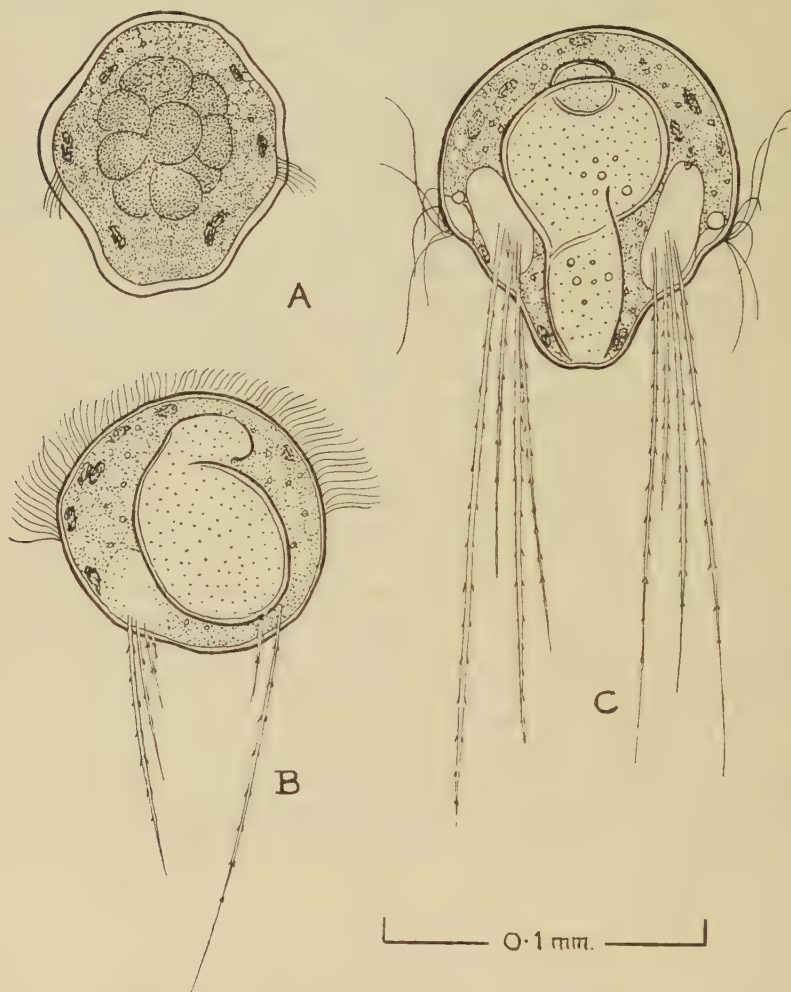


FIG. 1. Larval development of *Phragmatopoma californica*. A, trochophore, showing central mass of megameres and patches of pigment granules amongst the peripheral micromeres. B, C, young chaetigerous larvae, showing elaboration of the gut, chaetal sacs, and prototroch. B in dorsal view, C in optical section.

lateral region of the larva (fig. 1, B and C). The patches of yellow-brown pigment increase in number and are irregularly scattered throughout the ectodermal layer. At this stage a weak telotroch may also be seen and the bright red pigment of the eye-cups has appeared. The chaetae, which are from the first of the barbed type characteristic of all sabellariid larvae, rapidly

increase in number and when the larva is 36 hours old the single pair of chaetal sacs from which they arise may be clearly seen (fig. 1, c). Five or six chaetae are borne on each side in larvae two days old (fig. 2) but there may be as many as 30-40 long barbed chaetae on each side by the time the larva is ready to metamorphose. When three or four days old the larva has become clearly lop-sided, the head overhanging the mouth (figs. 2, 3), and this appearance is later accentuated by the development of the oral lappets (figs. 4, 5). By this time the telotroch is better developed and the 'grasping cilia' may also be seen. These are apparently formed by three or four adjacent cilia clinging together, and retain the barbed chaetae against the side of the body when swimming. These grasping cilia can release the barbed chaetae which are thrust outwards and forwards in response to abnoxious stimuli, or when braking (fig. 3). The telotroch becomes stronger as the larva develops and is often quite prominent in old planktonic larvae. The prototroch becomes strongly developed and the mouth, stomodaeum, and the regions of the oral lappets also become strongly ciliated. A pair of red eye-spots is usually recognizable by the time the larva is four days old, but in many larvae only one is formed at first, and this is always on the left as in fig. 3). The yellow-brown pigment-patches become grouped along the prototroch and telotroch and are scattered over the head and pygidium, but are never present on the segmental region of the body. Dark stellate chromatophores, on the other hand, appear in the trunk region. At first three rows may be recognized and these demarcate the three

segments usually known as the parathoracic segments of the adult (figs. 4, 5, 6). These three parathoracic segments are the first to be clearly delimited, the abdominal segments gradually becoming recognizable behind this region with increasing age (figs. 7, 8, 9). The segments anterior to this parathoracic region, with which we are chiefly concerned here, are peculiar in that they are not distinguishable until later in development.

Thus the part of the head overhanging the mouth or 'oral hood' consists of two tiers: (1) the prototrochal band, (2) the oral lappets on each side of the mouth. The mouth is bounded posteriorly by a U-shaped area which in later larvae may be recognized by its glandular nature to be the tube-building



— 0.1 mm —

FIG. 2. Dorso-lateral view of a young chaetigerous larva. The incomplete prototroch, the chaetal sacs and single eye-spot, and the first telotrochal cilia may be seen.

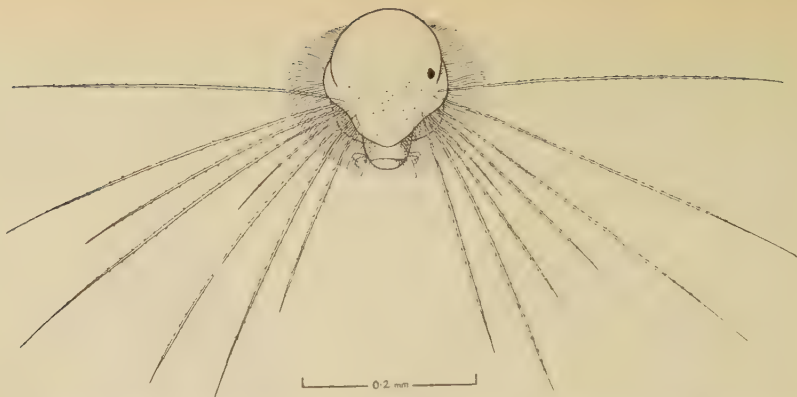


FIG. 3. Ventral view of a young larva with chaetae extended. Note the greater development of the prototroch, the telotroch with 'grasping cilia', and the oral lappets developing under the chaetal lobes.

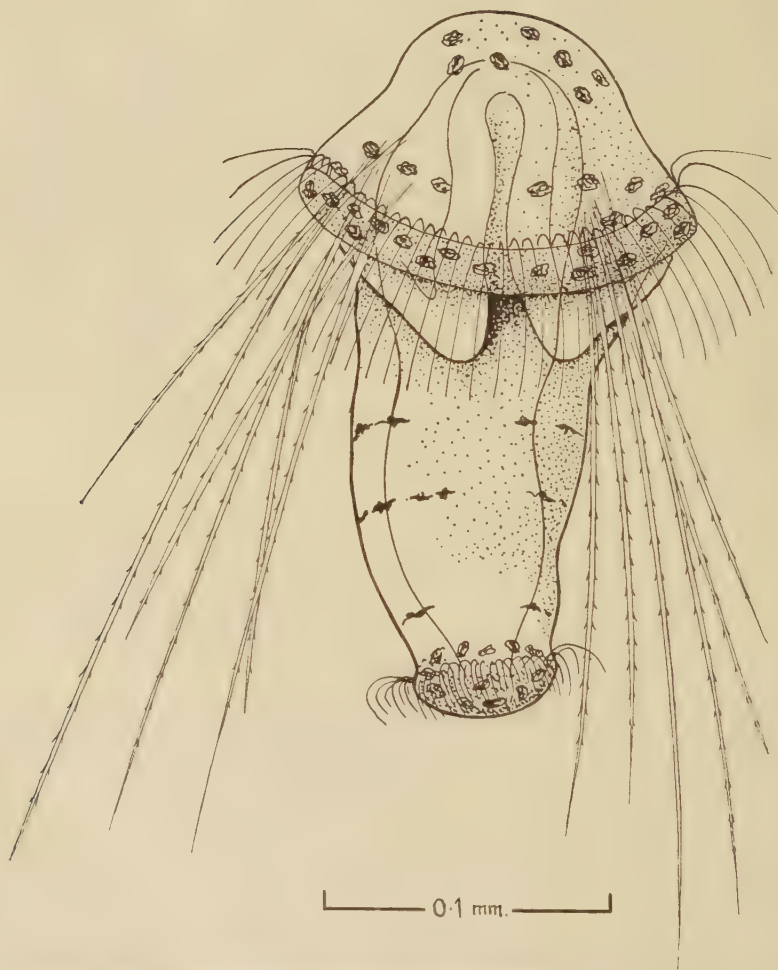


FIG. 4. Ventral view of a larva showing the development of the oral lappets. Note also the greater development of the telotroch, the distribution of pigment patches along the prototroch and non-segmental regions of the body, and the three lines of stellate chromatophores demarcating the future parathoracic segments.

organ of the adult (figs. 7, 10). The long barbed chaetae arise, as already noted, from a single pair of chaetal sacs, and these together with the building organ belong to the first segment. The lateral lips probably also belong to this segment, but it is not impossible that they represent true palps.

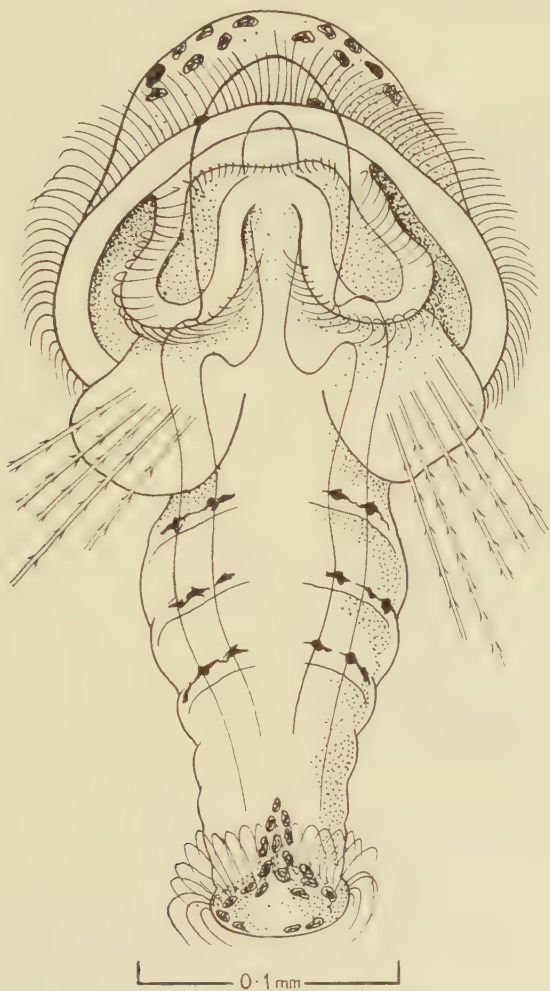


FIG. 5. A slightly later larva in postero-ventral view, showing the development of the ciliated oral lappets bordering the mouth under the rim of the hood bearing the prototroch. Note also the lobes bearing the barbed chaetae (only the bases of which have been drawn) and the further development of the trunk.

Before the building organ becomes clearly recognizable a pair of appendages may be seen growing out from the head in the dorso-lateral region just behind or at the end of the prototroch (figs. 6, 7, 8). It is difficult to homologize these appendages in the larva, but since in the adult they are innervated from the brain-mass they are presumably prostomial in origin, but in the

present state of our knowledge it is impossible to say whether they should be regarded as palps or antennae. When well developed they may be seen to have a ciliated groove communicating with the mouth. The groove passes over the

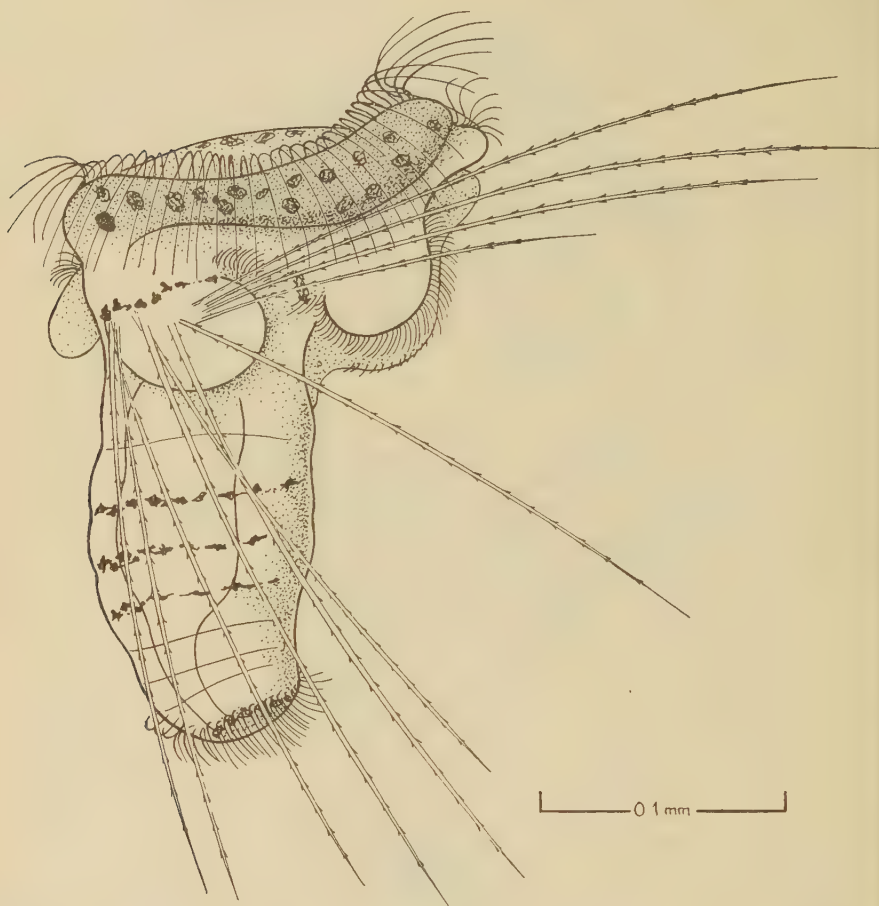


FIG. 6. Another larva slightly older than that in fig. 5, seen from the right side, showing the relation of the oral hood and prototroch, the ciliated lappets and the lobes bearing the barbed chaetae. A tentacle may be seen growing out in the dorsal region just posterior to the prototroch, and cilia leading from the base of the tentacle to the mouth may be seen developing over the chaetigerous lobes. Note the grasping cilia and demarcation of the first abdominal segments.

parapodial lobes bearing the barbed chaetae, and under the prototrochal band, conveying the phytoplankton on which the larva feeds into the mouth (figs. 7, 8). The yellow pigment-patches which have been noted to be characteristic of the asegmental regions of the body, appear on the distal half of each tentacle along the dorsal side, and this may be regarded as additional evidence that these appendages are in fact prostomial in origin (fig. 8).

As growth proceeds, the three parathoracic segments acquire sheaves of long spear-shaped chaetae characteristic of the adult, and large mobile feet bearing uncini grow out from the abdominal segments (figs. 8, 9). The complementary bundles of chaetae are not recognizable until later. In many larvae a single barbed chaeta may also be seen arising from the last parathoracic seg-

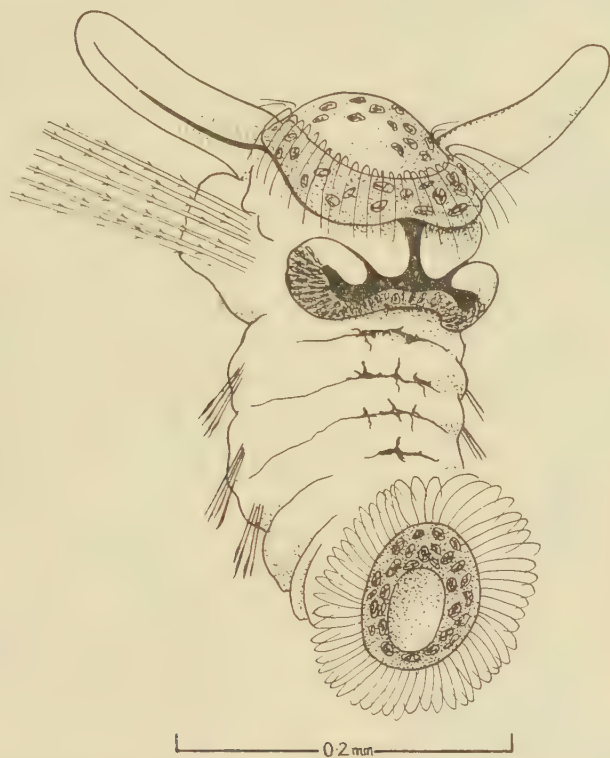


FIG. 7. A larva in ventro-lateral view, showing the further development of the prostomial tentacles and oral region. Notice the mid-ventral groove of the tentacle leading under the hood to the mouth, the U-shaped building organ (here buckled into a W owing to the flexure of the trunk), and the parathoracic chaetae.

ment (fig. 8). About the same time ventral cirri may be seen growing out from the first parapodial lobes, and soon afterwards the barbed chaetae begin to break off and fall out, gradually being replaced by flattened chaetae or paleae, simpler in structure than those of the adult. A segment between the first segment and the first parathoracic segment at last becomes apparent, a single sheaf of chaetae being protruded on each side about the time of settling (fig. 10).

Under natural conditions the larva probably settles out of the plankton as much as 2 months or even longer after the initiation of development. The barbed chaetae have by then been completely replaced by flattened paleae, and the prototroch and telotroch are lost. The caudal tail grows out and is

provided with a pair of dark patches of pigment probably acting as eye-spots, the young worm being able to walk backwards with the aid of the mobile legs

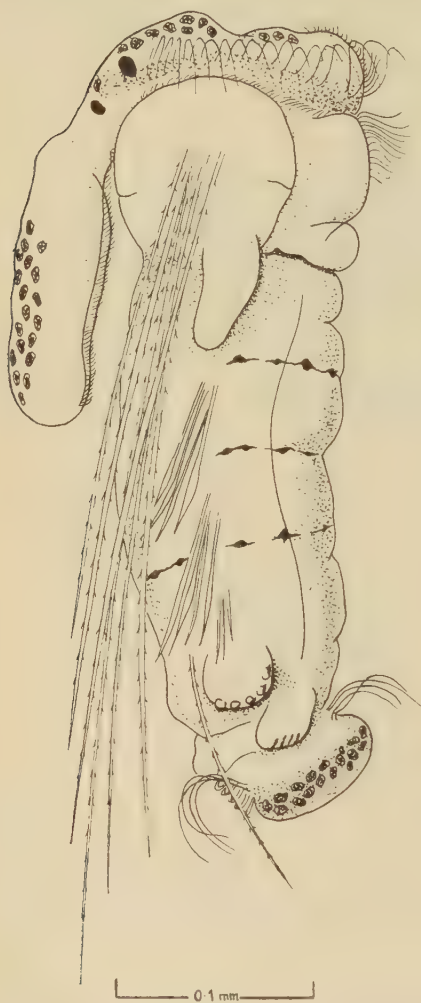


FIG. 8

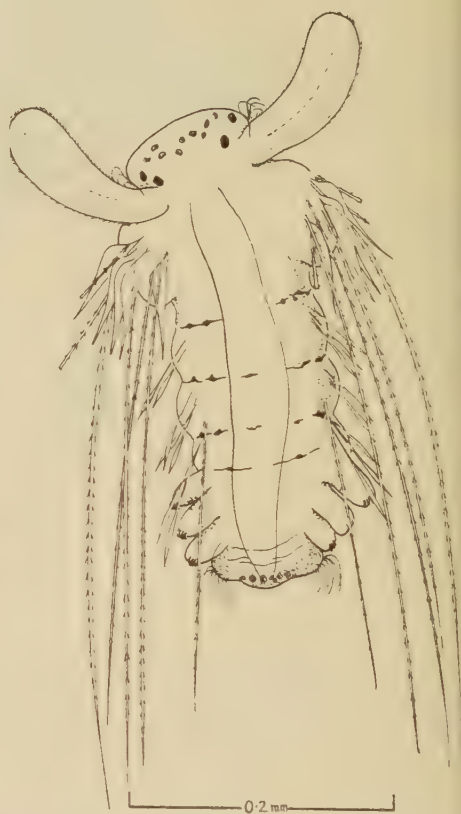


FIG. 9

FIG. 8. A slightly later larva seen from the right side, showing the ciliated groove of the prostomial tentacle passing to the mouth. The first parapodial lobes of the abdominal region bearing uncini, and the single barbed parathoracic chaeta may also be seen.

FIG. 9. A late planktonic larva in dorsal view, showing the replacement of the barbed chaetae by simple paleae, and the development of the three pairs of mobile parapodial lobes in the abdominal region.

representing the parapodia of the first three abdominal segments, moving about quite actively for a short time after settling. After a brief phase of such activity, tube building commences and the caudal tail becomes normally

reflexed. The three stages in the life-history are shown diagrammatically in fig. 11. The dorsal region of the first segment has by then begun to grow very rapidly so that the prostomium and the prostomial tentacles are pushed into a ventral position. This dorsal extension carries the paleae upwards and they eventually become arranged in an opercular crown on top of a thick stalk or peduncle. At the same time the ventral surface of this stalk becomes elaborated to form a system of feeding tentacles leading to the mouth.

Adult structure

In the adult the paleae become arranged to form an almost complete crown on top of the stalk, forming a very effective stopper to the tube when the animal retracts. On the ventral side a large number of tentacles are arranged in rows, and each with a ciliated groove conveys particles through collecting channels to the mouth (fig. 12). A sheaf of simple chaetae emerge in the ventral region on each side of the building organ, and a pair of small protrusions representing the notopodial cirri of the same segment may be seen in the corresponding dorsal region. A second pair of sheaves also emerging from the ventral region may be seen a little farther back, and a pair of rather better developed notopodial cirri may be seen in the corresponding dorsal region.

The opercular chaetae or paleae are arranged in three series of concentric and almost complete rings. They arise from two pairs of paleal sacs, the outer and middle rows from one, the inner row

from the other. The paleal sacs are embedded in the dorsal muscle of the most anterior segments and plunge right back into the second segment or the first parathoracic segment, one pair passing back slightly farther than the other. The paleae are produced continuously, the paleae from each sac fanning out into one portion of a hollow cone bisected by a plane through its axis, the paleae arranged in ascending age, the youngest towards the dorsal side. The apposition of the two pairs of paleal sheaves, one pair of which is concentric to the other, produces the virtually complete crown of the operculum, the paleae from above appearing to be arranged in concentric split-rings, the main break occurring where the new paleae are moving up



FIG. 10. A young bottom stage (probably *Sabellaria cementarum* Moore) in ventrolateral view, showing the early development of the oral tentacle system, and the three series of opercular paleae. The slightly annulated prostomial tentacle is clearly differentiated from the oral tentacles.

from beneath. The arrangement of the paleae and the structure of the anterior region is shown in figs. 12, 13, 14.

Thus in the adult *Phragmatopoma californica* there are two pairs of chaetal

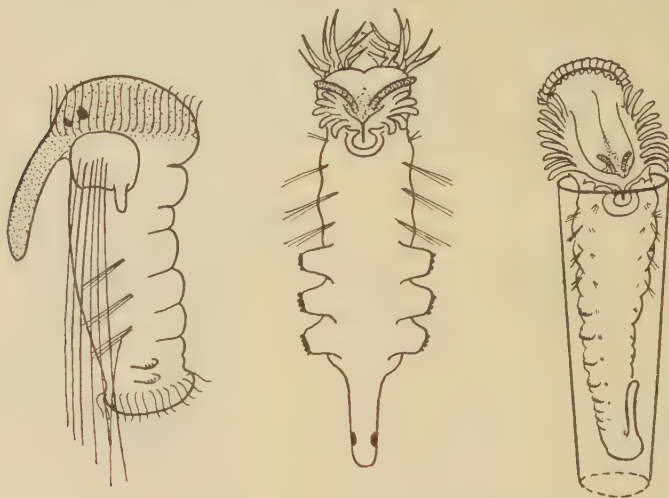


FIG. 11. Three phases in the career of an advanced sabellariid: A, planktonic stage; B, settling stage; C, adult stage. Prostomial tissue stippled.



FIG. 12. Structure of the anterior region of *Phragmatopoma californica*. A, ventro-lateral view, showing relation of the oral tentacle system on the ventral side of the opercular stalk to the mouth and prostomial tentacles (inset illustrates pattern of ciliary currents whereby food particles are conveyed to the mouth). B, dorso-lateral view showing the arrangement of the opercular paleae and the reduced dorsal cirri of the first two segments.

sheaves forming the opercular paleae, a small sheaf of simple chaetae emerging ventrally on each side near the building organ, and another similar pair emerging ventrally in the next segment. The following segment, which is the first parathoracic segment, possesses the usual arrangement of sheaves of chaetae.

Details of the nervous system will not be described here, since the detailed studies of Collis (1952) on *Sabellaria alveolata*, which agree with those of Johansson on the same species and with observations made by the present writer, will be appearing elsewhere in the press.

DISCUSSION

Many diverse views have been expressed regarding the origin and homologies of the opercular paleae. Most workers seem to agree that they are

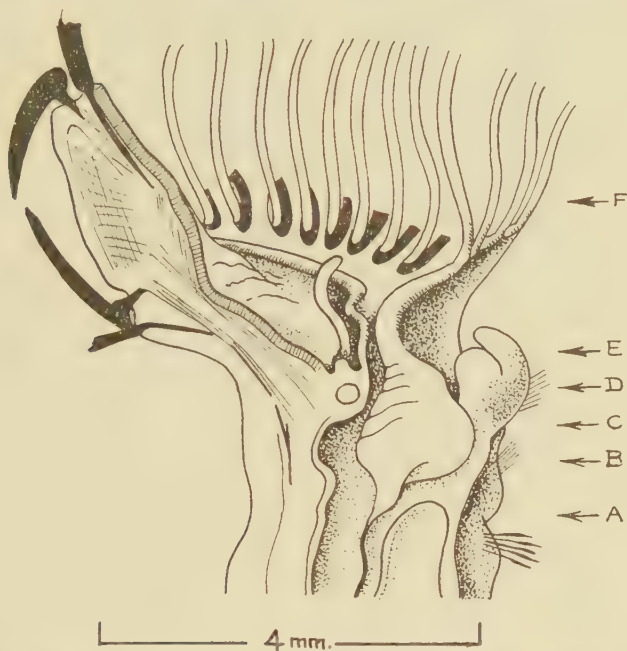


FIG. 13. Stereo-section of the anterior of *Phragmatopoma californica* to show the relationships of the operculum and opercular stalk, the oral tentacle system, and the prostomial tentacles. The letters refer to the approximate position of the sections shown in fig. 14.

derived from the first segment, but differ in opinion as to which the first segment really is. Some believe that although the paleae may be referred to a single segment, the opercular stalk may actually be formed from more than one segment. Quatrefages (1848), Grube (1877), Meyer (1887, 1888), Gravier (1909), McIntosh (1922), Fauvel (1927), Johansson (1927), and Hartman (1944), all agree that the opercular paleae belong mainly to one segment. Johansson, however, believes that there may be several segments anterior to this 'paleal segment' which have fused to the prostomium, and that they also may have contributed to the opercular stalk and to the series of paleae. Meyer and Gravier conclude that the operculum represents the notopodia of the first segment. Hartman concludes that the paleae represent both the notopodia and the neuropodia of a single segment, and that the heavy crotchets

which are present in the operculum of some genera (*Lygdamis* and *Idanthyrus*) represent the notopodia of another segment, the first small chaetal sheath emerging on each side of the building organ being the neurochaetae of this latter segment. The extra bundle of chaetae which she claims to be embedded in the opercular stalk of *Sabellaria* does not exist.

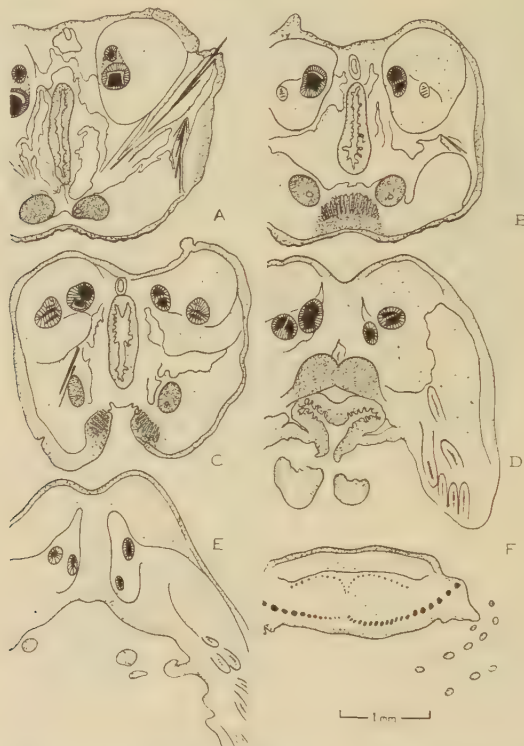


FIG. 14. Sections through the anterior region of an adult *Phragmatopoma californica*. A, through the 1st parathoracic segment, showing the spear-shaped chaetae emerging from the notopodium and arising near the ventral nerve cord, and the ventral chaetae arising dorsally. The chaetal sacs of the opercular paleae in this and following sections may be seen in the dorsal longitudinal muscle-blocks. B, through the 2nd thoracic segment, showing the base of ventral chaetae and the tissue of the building organ. C, 1st thoracic segment, through the building organ. D, through the mouth and cerebral ganglia. E, through the base of the opercular stalk. F, through the distal end of the opercular stalk, showing arrangement of paleae.

In both *Sabellaria* and *Phragmatopoma* the opercular paleae arise from two pairs of sacs, and although in *Sabellaria* the paleae are arranged in three rows, the middle and inner rows arise from a single sac (Watson, 1910 *a, b*; Hartman, 1944; Ebling, 1945). As the notochaetae and the neurochaetae of each segment arise each from a single sac it is reasonable to infer that the paleae represent either the neurochaetae and the notochaetae of a single segment, or the components of two segments. It has been seen that one paleal sac projects back very slightly more than the other in both *Sabellaria* and *Phragmatopoma*, and

also that the first two visible segments have one pair of sheaves only. The simplest possible explanation of the origin of the paleae is to refer them to the missing sheaves of these two segments. The question then arises whether these represent notochaetae or neurochaetae, but in the present state of our knowledge it is quite impossible to say to which they should be ascribed. It may be argued that they are clearly notochaetae because of their dorsal position, but on the other hand they show obvious resemblances to the parathoracic spear-shaped chaetae, which, although emerging in a dorso-lateral position, arise near the ventral nerve cord. The shorter chaetae which emerge in the ventro-lateral position in each of the first five segments arise in a more dorsal position, and this is clearly shown in fig. 14, A. This suggests that the notochaetae and neurochaetae are actually reversed, not only in the abdominal region, as Hartman has suggested, but throughout the segmental region of the body. On this hypothesis the opercular paleae and the spear-shaped parathoracic chaetae are thus all neurochaetae, and in support of this suggestion it may be pointed out that in less advanced families, large specialized chaetae tend to be characteristic of the neuropodium rather than the notopodium, and it is only in the most advanced families such as the Sabellidae and Serpulidae that uncini occur in the dorsal lobe in the anterior part of the body. Although sections of sabellariids do suggest that the neuro- and notochaetae have in fact become transposed, there is as yet no real evidence that such transposition has occurred either in this family or in the anterior region of sabellids and serpulids, and it is probably safer in these instances to call the chaetae which emerge from the notopodium, notochaetae, and from the neuropodium, neurochaetae, although further research may show that reversal has in fact taken place.

However, it seems quite clear that the paleae are derived from two segments, since if they represented the components of a single segment the existence of a further segment must be invoked, and there is no anatomical or developmental evidence for this assumption. Mention must also be made of a number of nerves running forwards from the circum-oesophageal commissures described in *Sabellaria alveolata* by Johansson and more recently by Collis, and it is the presence of these nerves which prompted Johansson to postulate the existence of as many as five segments in the operculum. Apart from these nerves, which may not necessarily be segmental in origin, the existence of further segments is not indicated by any other structure either in the larva or the adult as already noted.

The precocity of development of the first segment as compared with the delayed appearance of the following segment in the larvae of *Phragmatopoma californica* has already been emphasized, and the stalk of the operculum (as distinct from the opercular paleae) in the adult seems to be mainly contributed by the first segment. An evolutionary series in the elaboration of the operculum may be traced from *Cryptopomatus* through *Lygdamis* and *Sabellaria* to *Phragmatopoma* (fig. 15). In *Cryptopomatus* the operculum and the paleae are hardly developed. In *Phalacrostemma*, *Cryptopomatus*, *Lygdamis*, and

Idanthysus heavy hooked chaetae (crotchets or nuchal hooks) are also present in the opercular crown, but these seem to belong to the outer row of paleae of *Sabellaria* and *Phragmatopoma* and do not indicate the presence of another segment in the operculum. The prostomium which is hardly recognizable in the adult *Phragmatopoma* is more prominent in the more primitive genera. It is relatively large in *Cryptopomatus*, and in *Lygdamis* is produced into a process projecting forwards between the opercular lobes. In the higher genera the

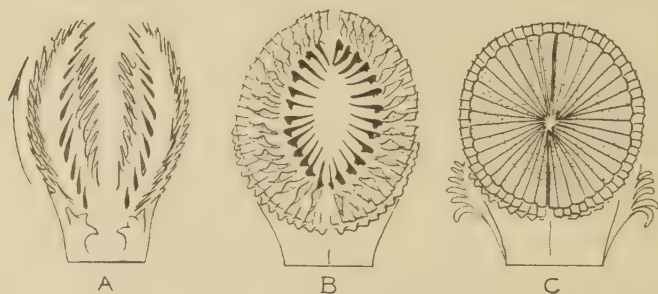


FIG. 15. Evolution of the opercular crown in Sabellariidae. A, *Lygdamis*; B, *Sabellaria*; C, *Phragmatopoma*. The arrow indicates the generation of the paleae, the youngest at the base of the stalk or dorsal. Inner row of paleae, black; middle row, stippled; outer row, unshaded.

prostomium is inseparably fused with the opercular stalk, and it is difficult to define its limits. However, the rows of eye-spots which run forwards in the mid-ventral line from the bases of the prostomial tentacles in *Sabellaria*, probably arise from prostomial tissue. The oral tentacles which are developed from the ventral side of the opercular stalk from the first segment are absent in *Phalacrostemma* and arranged in a relatively simple manner in such genera as *Lygdamis*, but are much more elaborate in *Sabellaria* and *Phragmatopoma*. For the synonymy of these genera the reader is referred to the work of Johansson (1927).

The single pair of prostomial tentacles have a feeding function in the larvae, and also in the adults of the most primitive genera such as *Phalacrostemma*, but are reduced in importance with the elaboration of the oral tentacle-system in the more advanced genera. The prostomial tentacles appear to be directly comparable to similar structures in the spionids and other related families, but it is proposed to discuss these relationships in another paper.

CONCLUSION

The constitution of the anterior region of the sabellariid worms may thus be interpreted as follows.

Prostomium. Usually reduced in the adult and partly or wholly concealed by the development of the operculum. In the larva, well developed and provided with one or two pairs of red eye-spots and a strongly developed prototroch. One pair of long tentacles with a ventral ciliated gutter communicating with the mouth and used for feeding arises from the end or just

anterior to the prototroch. In the adult these tentacles are important for feeding only in *Phalacrostemma*, but in most genera are probably mainly sensory.

Segment 1. Hypertrophied and constituting the main part of the opercular stalk. In the larva it is prominent and bears a pair of chaetal sacs from which long barbed chaetae protrude. These sacs correspond to one of the pairs of chaetal sacs which in the adult bear the opercular paleae—probably those giving rise to the inner and middle rows. All the opercular paleae correspond with the chaetae emerging from the notopodium of succeeding segments, and in the absence of further evidence may be referred to as notochaetae. The complementary sheaves are not recognizable in the larva; in the adult they protrude ventrally on each side of the building organ, which belongs mainly to this segment, although glandular tissue extends back into the second segment. The segment contains a normal arrangement of vascular and nephridial systems.

Segment 2. In the larva this segment is late in appearing, but in the adult it contains the normal complement of organ systems. One of the pairs of chaetal sheaves is represented by the outer whorl of opercular paleae, corresponding with the chaetae which emerge from the notopodium of succeeding segments, and may be regarded as notochaetae. The complementary sheaves are simple and emerge in a ventro-lateral position.

The following segment is the first parathoracic segment (in Hartman's terminology, and equivalent to Johansson's 'first paleal-bearing thoracic segment'); it bears a normal complement of organ systems, spear-shaped chaetae corresponding to the opercular paleae emerging dorsally, and simple chaetae emerging ventrally.

I am glad to have this opportunity of expressing my thanks to Professor Martin W. Johnson of the Scripps Institution of Oceanography, University of California, for every assistance in enabling the work on the larvae to be carried out during my stay at La Jolla, and to Dr. F. J. Ebling of the Department of Zoology of the University of Sheffield for kindly reading this paper in typescript, and for most useful criticism.

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Trimorphism in the Ichneumonid Parasite *Gelis corruptor*

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SUMMARY

1. The ichneumonid parasite *Gelis corruptor* is trimorphic in the development of wings: females are invariably apterous; males may be either macropterous or micropterous.

2. Females show no trace of wing-buds in the pupa and are without wing-disks in the late larval stage. The female aptery is a sex-limited character and is of genetic origin.

3. All male larvae have wing-disks and are potentially fully winged males. On a host providing adequate nourishment, the male larva develops into the macropterous form; on a host providing meagre nourishment, it develops into the micropterous form. Eggs or young larvae transferred from a small to a large host develop into macropterous males; larvae removed from large hosts before they have finished feeding develop into micropterous males. The dimorphism of the male sex is therefore controlled by the host viewed as a quantity of food.

4. The two forms of males differ not only in the development of the wings but also in the structure of the thorax, the size of the ocelli, the form of the endoskeleton, and the development, innervation, and tracheal supply of the thoracic muscles.

5. The trimorphism is strict, since all females are apterous and (with the exception of one abnormal specimen) all the males examined belong without intermediates to either the macropterous or the micropterous form.

6. The principal differences observed between the three forms all appear to be bound up with the condition of the wings. How the development of this complex of characters is organized, particularly in the two forms of males, presents an interesting problem of morphogenesis.

INTRODUCTION

IN two previous papers it was shown (1) that males of the parasite *Trichogramma semblidis* exist in two distinct forms, one winged, the other wingless; and (2) that which of the two forms shall appear in a particular case is determined by the species of host on which the individual parasite is reared (Salt, 1937, 1939). Those observations raise several problems of morphogenesis. At what stage in its development is the form of an individual male determined? Have the two forms a different genetic constitution? If not, what factors, precisely, determine the phenotype, and what morphogenetic processes take place as the determination is effected?

These and related problems cannot be conveniently attacked by using *T. semblidis*. It is a parasite of insect eggs and is only 0.5 mm. long when full grown. It is an internal parasite and its immature stages cannot be seen without opening the host, thus upsetting the development it is desired to observe.

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For these reasons *T. semblidis* is unsuitable for experimental morphology. Clearly an investigation of the morphogenetic aspects of this kind of dimorphism would have more chance of success if a similar example could be found in a larger animal with habits such that its development could be observed and experimentally manipulated. Search for such an example was considered in 1939 but could not be actually initiated until after the war, when it led to the investigation now reported.

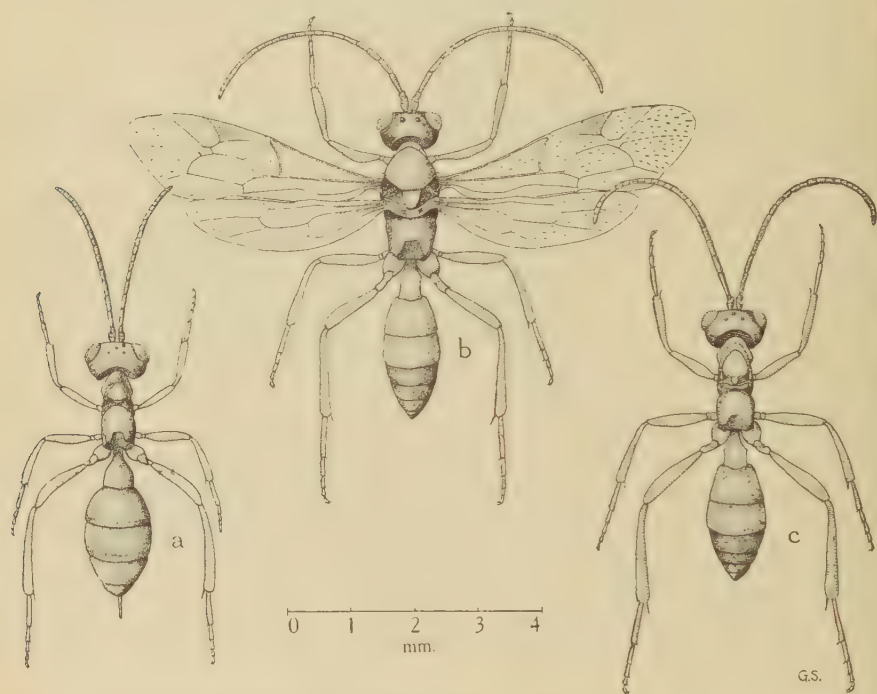


FIG. 1. Habitus sketches of the three forms of *Gelis corruptor*. a, female; b macropterous male; c, micropterous male.

Material that appears to be suitable for an attempt on the problem has been found in *Gelis corruptor* (Grav.). This is an ichneumonid hyper-parasite which reaches a maximum larval length of 5.5 mm. and which feeds externally upon its host. The females are always completely apterous; the males will be shown below to exist in two distinct forms, which differ most conspicuously in the state of the wings. One form is macropterous and is capable of sustained flight; the other has rudimentary wings so small that its condition is well described as micropterous. The species can therefore be said to have three phenotypes, the apterous females and the micropterous and macropterous males, of very distinct appearance (fig. 1).

It is proposed in this paper first to describe the three states of the wing development in *G. corruptor*; secondly to trace the origins of the trimorphism;

and thirdly to show the extent and the constancy of the differences between the three forms. A second paper is projected in which the morphogenesis of the different forms will be described.

MATERIAL AND METHODS

There are many references in the literature to the rearing of species of *Gelis* from hosts collected in nature, but I cannot find that any species of the genus has hitherto been bred through successive generations in the laboratory. It is therefore necessary to describe the methods used.

The breeding of *G. corruptor* is made laborious by the fact that it is a hyper-parasite. The maintenance of a strain for experimental purposes therefore involves keeping cultures of at least two other animals, a primary parasite which the *Gelis* will attack and a host on which the primary parasite can be reared. A parasite and host complex suitable for continuous laboratory culture is that of the ichneumonid *Nemeritis canescens* (Grav.) and the flour moth *Ephestia kuehniella*. The moths lay readily if they are put into a jar with rolled oats, on which their larvae feed. Stock cultures of *Nemeritis* can be maintained by introducing adult parasites into the jars when the *Ephestia* larvae are nearly full grown.

Nemeritis suitable for parasitization by *Gelis* are better obtained from special cultures. About 200 *Ephestia* larvae between 20 and 24 days old at 25° C. are put into a large jar with only a very small quantity of rolled oats sprinkled less than $\frac{1}{4}$ in. deep on the bottom. After a day or so, when the caterpillars have formed silken tubes and cocoons in which to live and to pupate, a dozen adult *Nemeritis* are introduced. They reach the caterpillars with their ovipositor, and lay their eggs inside them. The *Nemeritis* larva completes its feeding 11 days later and then forms its own cocoon inside that of the *Ephestia*. On the fourteenth day the jar can be emptied and the rolled oats and *Ephestia* silk carefully stripped from the grey *Nemeritis* cocoons. These now contain *Nemeritis* larvae and pre-pupae which are suitable hosts for *Gelis*. The cocoons can be conveniently handled and used when eight of them are glued on a piece of cardboard $2 \times \frac{7}{8}$ in. Such cards of hosts can be stored for a short while at 8° C., but *Nemeritis* is intolerant of refrigeration or of long delay in its pupal development, and hosts kept for more than two or three weeks are usually unsuitable for parasitism.

Gelis adults can be kept in glass tubes 1 in. in diameter and $4\frac{1}{2}$ in. long, closed at one end with cotton wool and at the other end with a cork to which a small piece of raisin can be pinned as food. They live for several weeks at temperatures between 20° and 27° C. provided that a high relative humidity is maintained. For parasitization, a card of *Nemeritis* cocoons is put into the tube. The *Gelis* pushes her ovipositor through a cocoon, paralyses the host, and lays her egg on or beside it. The *Gelis* larva lives externally on the paralysed host, perforating its cuticle and feeding at first on its fluid contents until only the skin and hard parts are left. These are eventually pushed to one side of the cocoon, inside which the *Gelis* larva spins a thin cocoon of its own.

There it pupates and thence it escapes, when it has become adult, by biting a hole through its own and the host cocoon.

In the present investigation a complete account of the reproduction of each *Gelis* was required. The females were therefore invariably kept in separate tubes, and the cards of hosts that had been exposed to them were removed to separate vials for emergence of the progeny. About a week after the first emergence from a card, each host cocoon that remained intact was opened and examined to discover whether any *Gelis* had died in an immature stage. During this examination the condition of the unparasitized hosts was noted so as to provide an indication of their suitability for parasitism. In this way a complete account of the reproduction of each individual female has been obtained and, since each adult *Gelis* was identified by a number and since mating was restricted, the ancestry and progeny of every member of the strain are known.

Although *Nemeritis* is especially useful as a host because it can be made available throughout the year, other Hymenoptera are also suitable. In the work here to be reported, larvae of *Apanteles glomeratus* (Linn.) and of two species of *Lasius*, *L. flavus* (Fabr.) and *L. niger* (Linn.), were also used. The *Apanteles* were obtained from caterpillars of *Pieris brassicae* collected in nature in September and brought to maturity on cabbage leaves in the laboratory. The yellow *Apanteles* cocoons are formed in clumps but can easily be separated and glued in rows on thin cardboard. If they are then kept at a temperature near 0° C., the larvae and pupae within them remain suitable for parasitization by *Gelis* for several months or even as long as a year. It has been found that cocoons containing larvae or pupae of queens of *Lasius flavus* or *L. niger* can be used as hosts for *G. corruptor*. They are obtainable in nature in June and can be stored for some weeks at low temperatures, but after a month or two an increasing proportion of them becomes unsuitable.

Females of *Gelis* can be collected in the field at any time of the year but not all of them give rise to a strain. Of 43 wild females set up in the manner described only 22 left any progeny, and to the present only 14 strains have been established. This paper is concerned entirely with one species, *G. corruptor*, and almost exclusively with a particular strain (my number 456). The original female was collected and kindly sent to me by Rev. E. J. Pearce, C.R., from Symondshyde Wood, Welwyn, Herts., on 25 August 1945; it gave rise to 14 filial generations before my going abroad in 1948 made it necessary to bring that strain to an end.

THE TRIMORPHISM OF THE WINGS

(a) *In the adult stage*

The wings of the macropterous male are perfectly formed (fig. 1), and are as large in proportion to the size of the animal as are the wings in related Cryptine genera. In order to substantiate this statement, the length of the forewing and the length of the thorax were measured in five males of each of

four species. The following wing/thorax ratios were found: *Hemiteles hemipterus* 2:4; *Leptocryptus bellulus* 2:5; *Gelis corruptor* 2:6; *Phygadeuon laevis* 2:7. Sustained flight of macropterous males was frequently observed.

In the micropterous male the wings are represented by small sac-like lobes, the anterior pair only about twice as long as the tegulae at their bases; the posterior pair less than half as long as the anterior (fig. 2). These rudimentary wings are membranous, pale, and translucent, and are fringed with short hairs at their tips. On several occasions micropterous males were observed to

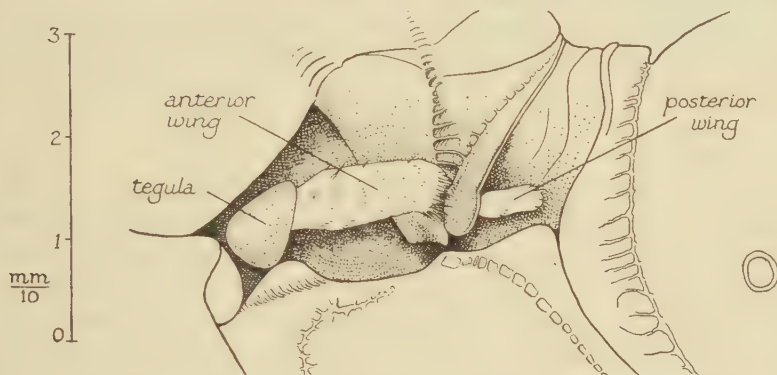


FIG. 2. Latero-dorsal view of part of the thorax of a micropterous male, to show the rudimentary wings.

move these tiny flaps up and down at a rate estimated at three or four times a second.

All the males of *G. corruptor* examined in the course of this work have at least rudimentary wings. Of the principal strain on which this paper is based (strain 456), 726 adult males have been bred, 277 of them fully winged and 448 micropterous. One male only does not fall clearly into either group. This abnormal specimen, to be described in more detail later, has enlarged lobes on the left side and partly developed wings on the right side. Other strains of *G. corruptor* bred in the course of this work have produced a total of 230 adult males. Of those, 91 are macropterous and 139 micropterous. No male of this species has been seen, or of any other species of *Gelis*, which did not have wings or the rudiments of wings—the point is emphasized because micropterous males like these are almost always described in systematic literature as ‘apterous’.

Adult females, on the other hand, show no trace of wings. No tegulae, axillary sclerites, or any other structures accessory to wings can be distinguished in this sex; indeed, the pleural and tergal sclerites are fused firmly together, so that even the space from which wings could arise is obliterated. Of the principal strain, 143 adult females have been bred; and of other strains of *G. corruptor*, 60 females. All of these, without exception, are in this completely apterous condition.

(b) In the pupae

The male pupae of *G. corruptor*, distinguishable from the female by the genitalia, invariably have wing-cases. In those pupae that will develop into macropterous males, the wing-cases are long and extend postero-ventrally, around the pleura as far as the middle of the metathoracic coxae. In the pupae that will develop into micropterous males, the wing-cases are small lobes of much the same size as the rudimentary wings of the adult, and are almost hidden in side view by the tibio-femoral joints of the meso- and metathoracic legs. No male pupa has been seen which did not have clearly recognizable wing-cases of one of these two kinds. The female pupae, in which the parts of the ovipositor are turned dorsally over the tip of the abdomen, have no wing-cases.

(c) In the larvae

In order to study the condition of the wing-disks in larvae of the different forms, it is first necessary to be able to distinguish larvae of the two sexes. With older larvae of *G. corruptor* this can be done by examining the posterior ventral surface. The female larva has a pair of small tubercles near the mid-ventral line on the eighth abdominal segment. In male larvae, the eighth abdominal sternum has no tubercles. Since this external examination can be made without injury to the larva, identification of the sex by this means can be corroborated by rearing the larva to the adult stage. When male and female larvae have been distinguished in this way, it is found that wing-buds are invariably present on the sides of the meso- and metathoracic segments of the male larvae but that no wing-buds can be seen on female larvae.

The reproduction of *G. corruptor* affords a better way of obtaining larvae that are certainly male. In this species, as in many other Ichneumonidae, fertilized eggs give rise to females, unfertilized eggs to males. In the course of this work, 192 adult progeny have been reared from 19 females known to have been unmated, and those progeny are all males. Following this evidence of arrhenotoky, larvae that are certainly male can be obtained at all stages of their development by rearing them from the eggs of virgin females. Examination of such larvae, by external observation in the later stages and by means of sections in the earlier stages, invariably reveals the presence of imaginal disks of the wings.

It is more difficult to obtain small larvae that are certainly female. The identification could presumably be made by the histology of the imaginal disks of the ovaries; but that has not been attempted. Instead, evidence that females of *Gelis* lack wing-disks in the early larval stages has been sought by use of another species, *G. instabilis* Först. This species reproduces by thelytokous parthenogenesis. A strain of it has been reared in the course of this work through 25 exclusively female generations, in which all of the 325 adults bred are females. Larvae of this strain, therefore, can be accepted as female. Small larvae about 1.5 mm. in length have been sectioned, as well

as mature larvae, and, although the leg-disks of the small larvae are easily seen, in no case have imaginal disks of wings been found. This evidence from *G. instabilis* lends support to the observations made on mature larvae of *G. corruptor*, and suggests that female larvae in this genus lack the imaginal disks of wings, at any rate from an early larval stage.

(d) Conclusions

From the foregoing observations two conclusions can be drawn. (1) The female sex of *G. corruptor* is completely without wings or wing rudiments in the adult, pupal, and late larval stages, and is probably without them in the early larval stages. (2) The male sex, on the other hand, is essentially a winged insect; the wing-disks that are invariably present in the male larva may not develop into full-sized wings capable of flight, but are always represented at least by rudimentary wings in the adult.

THE ORIGINS OF THE TRIMORPHISM

(a) Origin of the female aptery

No female *G. corruptor* has ever been observed that had wings or the rudiments of wings. This sweeping statement is based only in part on the 203 female adults bred and about 50 others collected in nature in the course of the present investigation. It is really based on a tautology. The genus *Gelis* is defined as having apterous females; and if the many systematists and collectors who have examined these insects have ever handled a winged female, they have necessarily placed it elsewhere in the classification. If winged females of *Gelis* exist, they can be discovered only by breeding them from the same parents as the normal apterous females; and I have found no reference to work other than that here being recorded on the breeding (as distinct from the rearing) of *Gelis* females.

Since, then, no female *G. corruptor* has ever been observed that had wings or wing rudiments, and no male has been observed that was without them, the presence or absence of these organs must be accepted as a sex-limited character determined, with the sex of the individual, by its genetic constitution.

Now the great majority of insects related to *Gelis*, within the whole of the Ichneumonidae or within the sub-family Cryptinae, are winged in both sexes. It is impossible, on both logical and biological grounds, to suppose that in a genus ancestral to *Gelis* the wings were lost in both sexes and then in the modern genus were redeveloped by the male. It must therefore be assumed that wings were originally present in both sexes and have been lost only by the female. In looking for the origins of the trimorphism of *G. corruptor*, then, it can be concluded that the aptery of the female is due to a genetic alteration having effect only on that sex.

(b) Origin of the male dimorphism

Since wing disks are invariably present in the male larvae of *G. corruptor*, it is necessary to consider what factors determine whether they shall develop

into the macropterous or the micropterous condition in the adult. Genetic and environmental factors have both to be taken into account. Of the latter, physical factors can be disregarded after the statement that the two forms were regularly bred side by side under identical conditions so far as the physical environment was concerned. Biotic factors, on the other hand, loom large in this problem, since the environment of a parasite is largely provided by its host.

(1) *The hosts.* The 726 males of strain 456 that were brought to the adult stage in the course of this work were all reared on one of four hosts: *Apanteles glomeratus*, *Nemeritis canescens*, and queen cocoons of the two ants *Lasius flavus* and *L. niger*. The last two need not be distinguished for our present purpose. On *Apanteles*, 372 males were reared; all, without exception, were micropterous males. On *Nemeritis*, 222 males were reared, 49 of them micropterous and 173 macropterous. On queen cocoons of *Lasius*, 132 males were reared, 27 micropterous, 104 macropterous, and the one asymmetrical specimen already mentioned.

These three hosts differ markedly in size. The average weight of ten resting larvae and prepupae of *Apanteles* was 4.1 mg., and none weighed as much as 6 mg. Ten *Nemeritis* had a mean weight of 10.8 mg. with a range from 8.6 to 12.6 mg. Twenty *Lasius* weighed on the average 16.5 mg., none being lighter than 13 mg.

It will be observed that all the male *Gelis* reared on *Apanteles*, the smallest host, were micropterous. This immediately suggests that the micropterous condition may be due to under-nourishment. The suggestion is supported by the fact that 78 per cent. of the males reared on the larger host *Nemeritis* were macropterous.

But on this hypothesis, if *Nemeritis* hosts weighing at most 12.6 mg. could usually produce macropterous males, *Lasius* hosts weighing at least 13 mg. should always have produced them. In fact, 20 per cent. of the males reared on *Lasius* were micropterous. This apparent discrepancy may be explained as follows. Queen cocoons of these ants were obtainable in nature only in June, but they were stored at a temperature of 4° C. and were used as long as a year later. *Gelis* males reared on fresh *Lasius* larvae invariably developed into macropterous males. It was only when the condition of the hosts had deteriorated that micropterous males appeared. In 11 cases, after a micropterous male had emerged from a *Lasius* cocoon, the cocoon was opened and it was found that the bulky remains of the host were putrified or mouldy. In the other 16 cases, although the actual host was not examined, the records show that other cocoons on the same card contained hosts that were unsuitable, either in being too far developed or in being unhealthy. There can be little doubt that these micropterous males reared on elderly *Lasius* did not find enough suitable food and, in spite of the bulk of their host, were partially starved.

That *Nemeritis* produced both macropterous and micropterous *Gelis* was due partly to the same cause. Resting larvae and prepupae of *Nemeritis* are

intolerant of storage at low temperatures and die in a few weeks. Owing to the demands of several different strains, it was sometimes necessary to use cocoons that had been stored a long while, in some of which the hosts would not be entirely suitable as food. In 37 of the 49 cases where micropterous males emerged from *Nemeritis* cocoons, there is evidence that the hosts were unsatisfactory. But micropterous males occasionally emerged from fresh *Nemeritis*. The explanation of these cases lies in the wide range of weight of this host, a range extending at least from 8.6 to 12.6 mg. Some of the smallest *Nemeritis* are not large enough to support the development of a macropterous *Gelis*, and a few micropterous males may emerge from a collection of *Nemeritis* cocoons however fresh the hosts.

(2) *The size of macropterous and micropterous males.* The hypothesis that the micropterous condition is due to under-nourishment of the male larva receives strong support from a comparison of the size of macropterous and micropterous adults. The comparison can best be made on dead specimens by measuring the length of the thoracic region, from the anterior margin of the pronotum to the end of the propodeum, because the inclination of the head and the degree of extension of the abdominal segments upsets the measurements of total length. Males of each kind have been measured by means of a micrometer eyepiece dividing the field into units representing 0.105 mm. Twenty-five macropterous males gave an average measurement of 19.7 units with a range from 17 to 22 units; twenty-five micropterous males gave an average measurement of 11.8 units, with a range from 6 to 15.5 units. The smallest macropterous males to be found in the whole collection measured no less than 17 units and the largest micropterous males that could be found measured no more than 15.5 units. There was, then, no overlap in the range of thoracic size of the two forms. It is interesting to notice, however, that the asymmetrical specimen mentioned above, having enlarged lobes on one side and partly developed wings on the other, falls between the size ranges of the normal forms. It has a thoracic length of 16.5 units.

(3) *The progeny of individual females.* In the routine maintenance of the strains, individual females were sometimes provided with one species of host at one time and a different species at another. From the records of the cultures it is therefore possible to consider the male progeny of individual females in relation to the kind of host on which they were reared. Ten cases are listed in Table 1. In these and in other cases, the male progeny of individual females were micropterous when reared on *Apanteles* and mainly macropterous when reared on the larger hosts *Nemeritis* or *Lasius*. It is clear, therefore, that one type of male does not derive from certain females and the other type of male from other females, but that each female can lay eggs capable of developing into both types of male.

(4) *The transfer of eggs and larvae.* The hypothesis that the dimorphism of the male *G. corruptor* has a genetic origin would imply that the females lay two different kinds of male eggs, one capable of developing only into the micropterous, the other only into the macropterous form. There is a certain *a priori*

attraction in this hypothesis since, in order to bring the sex determination of many Hymenoptera (haploid male—diploid female) into conformity with the idea of genic balance, Whiting (1933) has suggested that the haploid males are genetically of two types.

TABLE I. *The male progeny, reared on different hosts, of ten females of Gelis corruptor*

Parent	Number of male progeny on					
	<i>Apanteles</i>		<i>Nemeritis</i>		<i>Lasius</i>	
	macropt.	micropt.	macropt.	micropt.	macropt.	micropt.
A ♀7	..	6	3	1	3	..
H ³ ♀1	..	8	5	..	..	..
J ⁷ ♀4	..	12	4	..	..	..
J ⁷ ♀5	..	9	10	..	..	..
J ⁷ ♀7	..	13	4	..	..	..
J ⁷ ♀8	..	6	5	..	..	..
K ³ ♀1	..	1	6	..	..	..
K ¹¹ ♀4	..	5	5	1	..	..
L ¹ ♀2	..	3	..	..	9	..
L ¹¹ ♀1	..	1	1	..	5	1

The hypothesis of two kinds of male eggs developing separately into micropterous and macropterous males would involve a further assumption. It has been recorded above that 372 micropterous and no macropterous males emerged from *Apanteles*; 76 micropterous and 277 macropterous from *Nemeritis* and *Lasius*. If, therefore, there are two kinds of male eggs, either the female *Gelis* must be able to control their distribution, and must lay exclusively the micropterous type on *Apanteles* and mainly, though not exclusively, the macropterous type on *Nemeritis* and *Lasius*; or else, if the two types of eggs are laid at random, all eggs of the macropterous type laid on *Apanteles* and most of the eggs of the micropterous type laid on *Nemeritis* and *Lasius* must fail to develop on those hosts.

The second assumption is shown to be false by the routine breeding records. Throughout this work every host that did not give rise to a parasite has been examined before it was thrown away. In only a few cases, and usually where the host had clearly been unsuitable as food, were shrivelled eggs or dead larvae of *Gelis* found. The great bulk of the observations, some thousands in number, refute any suggestion that a large number of eggs, capable of developing only into macropterous males, were laid on *Apanteles* but failed to live on that host, or that many eggs capable of developing only into micropterous males died on *Nemeritis* and *Lasius*.

The first assumption can be examined experimentally. Eggs or young larvae laid on a small host can be transferred to a large host to see whether they develop into micropterous males even in the presence of ample food. Conversely, larvae feeding on large hosts can be deprived of their host before

they have finished feeding in order to discover whether they develop nevertheless into macropterous males.

For the purpose of the first experiment, two female *Gelis* known to be un-mated were isolated in vials and were provided with cocoons of *Apanteles*. After 2 days in one case and 4 days in the other, each host cocoon was opened. Three eggs and 11 young larvae were thus obtained, seven from each female. Fourteen *Nemeritis* larvae were removed from their cocoons and were killed by heating on a hot plate at 62° C. for 1 minute. They were replaced in their cocoons, and one of the *Gelis* eggs or larvae was put on each. In two cases the manipulations were clumsy and both the parasite and its host had to be handled twice. In both of these cases the male adult that eventually emerged was micropterous; in all the other twelve cases perfect macropterous males emerged. From the extensive rearing records already reported it is certain that none of these fourteen males would have developed into the macropterous form if they had been left on their original *Apanteles* hosts. It can therefore be concluded that twelve individuals destined to develop into micropterous males were induced to develop into the macropterous form by transference from the smaller to the larger host.

For the purpose of the second experiment, six female *Gelis* were each put with cocoons containing large, fresh larvae of *Nemeritis*, on which macropterous males would be expected to develop. After periods of 6 to 9 days in different cases, the cocoons were opened and nineteen *Gelis* larvae were found. These were measured and then replaced in the empty *Nemeritis* cocoons from which all that remained of the hosts had been removed. There they were allowed to continue their development without further feeding. Four larvae, which measured 1.6, 2.2, 2.8, and 3.2 mm. in length respectively, died. Five larvae, already 4.7, 4.8, 4.8, 4.8, and 5.2 mm. long respectively when found, developed into macropterous males. All of these were recorded as having finished feeding, and one of the three that were 4.8 mm. long had already begun to spin its cocoon. Ten larvae, which were between 2.6 and 4.4 mm. in length when deprived of their hosts, developed into micropterous males. In all of these ten cases the *Gelis* larva was still feeding when it was found, and a good deal of the host remained to be eaten when it was taken away. There is no reason to doubt that the last ten larvae would have developed into macropterous males if they had been left to complete their feeding, and their development to micropterous males must be attributed to the starvation that followed the removal of their hosts.

These two experiments demonstrate clearly that females of *Gelis* do not lay on *Apanteles* eggs that can develop only into micropterous males, and on larger hosts eggs that can develop only into the macropterous form. Together with the facts reported in the third paragraph of this section, they lead to the definite conclusion that the two male forms of *G. corruptor* do not develop from two different kinds of eggs but that, on the contrary, each egg can develop into either type of male.

(5) *Conclusions.* The four lines of evidence now recorded lead to a clear

conclusion about the origin of the male dimorphism. Both forms of male can be derived from the same female parent, and either form can develop from a particular male egg. Therefore the dimorphism has not a genetic origin. Micropterous males are smaller than macropterous males. Only micropterous males develop from the small host *Apanteles*, mainly macropterous males from the larger hosts *Nemeritis* and *Lasius*; and the exceptions in the last cases are explained by the unsuitability of some of the larger hosts as food. Eggs and young larvae transferred from *Apanteles* to *Nemeritis* gave macropterous males; larvae on *Nemeritis* that were deprived of their hosts before they had finished feeding developed into micropterous males. The conclusion is inescapable that which form of the male shall appear is determined by the host viewed as a quantity of food.

THE EXTENT OF THE TRIMORPHISM

The three forms of *G. corruptor* have been defined in the preceding pages with reference exclusively to the degree of development of the wings. But one cannot suppose the trimorphism to be restricted to the presence or absence of wings or wing-rudiments, for wings imply muscles, and muscles require phragmata and nerves and tracheae. In this section, therefore, it is proposed to inquire into the extent of the differences between the three forms.

The comparison is complicated by the fact that the individuals differ in two other general characters—in sex and in size. This paper is not concerned with secondary sexual characters as such. The female must be described, as well as the two male forms, because interest attaches to the question how far the flightless micropterous male may resemble the wingless female; but sexual differences that are usual in the Cryptinae need not be considered. Structural differences due to size have also to be taken into account, because the individuals bred in a single strain range in length from 1.9 to 6.0 mm. In order to eliminate differences merely of size, or due directly to size, the comparison that follows was made in the first instance by using a group of specimens between 3.5 and 5.0 mm. in length. But each detail has also been examined in the smallest and the largest individuals and, where no qualification is made, the differences described are to be understood as referring to the whole range.

(a) External structures

(1) *The thorax and propodeum.* At first sight this region of the body presents an appearance of quite different structure in the three forms (figs. 3 and 4), but closer examination reveals that the differences are largely due to alterations in one segment, the mesothoracic. In a dorsal view, the mesoscutum of the macropterous male (fig. 3, *a*) is so broad and so strongly convex that it hides most of the prothorax. The scutellum is correspondingly well developed. The micropterous male (fig. 3, *b*) is much more slender. Its mesoscutum is shorter, narrower, and less convex, so that a good deal of the pronotum is visible

laterally and in front. The scutellum, axillae, and metanotum are also greatly contracted. In the female (fig. 3, *c*) the development of the mesothorax is still further reduced. The scutum is short and flat, the scutellum a mere shelf, and the metanotum scarcely distinguishable beneath it.

The development of the mesoscutum in the macropterous male is also shown in a lateral view (fig. 4, *a*), which makes plain the convexity of the sclerite and the way it arches forward over the pronotum. In this view, the mesopleuron is seen to be correspondingly large: it is so wide and swollen that its surface makes up a large proportion of the thoracic wall. A side view

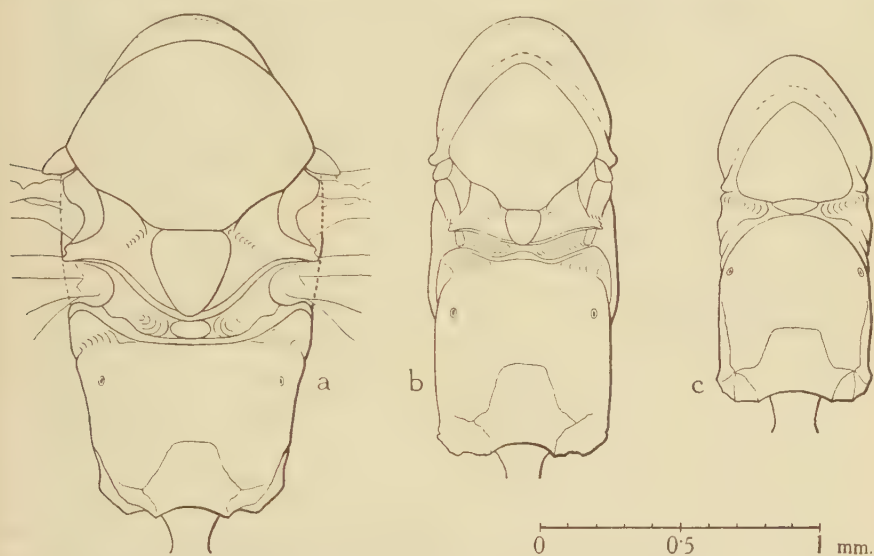


FIG. 3. Dorsal view of the thorax and propodeum of *a*, macropterous male; *b*, micropterous male; *c*, female.

of the micropterous male (fig. 4, *b*) emphasizes the flatness of its mesoscutum and shows that the mesopleuron also is shorter, flatter, and more upright in position. In the female (fig. 4, *c*) the mesopleuron is still shorter, especially dorsally, where it is fused directly to the notum.

A comparison of the thoracic structure of the three forms with that of the winged females and males of related genera shows that it is the apterous female and the micropterous male of *G. corruptor* that are altered from the normal. A side view of the thorax and propodeum in the winged female of *Hemiteles subzonatus* is shown in fig. 4, *d*; and it can be seen that the parts of the thorax have essentially the same proportions as those of the macropterous male *Gelis*.

The legs of females of *G. corruptor* are clearly stouter than those of the males. The legs of micropterous males also appear to be thicker in proportion to their length than those of macropterous males, the coxae especially seeming shorter and more swollen, but a detailed biometrical study would be required

to show that any such differences are not associated with the range of size rather than with the dimorphism.

(2) *The head.* The most striking difference between the heads of the two male forms is in the development of the ocelli. In the macropterous male they are very large and prominent. A dorsal view (fig. 5, *a*) shows that the posterior ocelli are only about their own diameter distant from the compound eyes, and less than twice their diameter apart; a frontal view (fig. 5, *b*) shows how

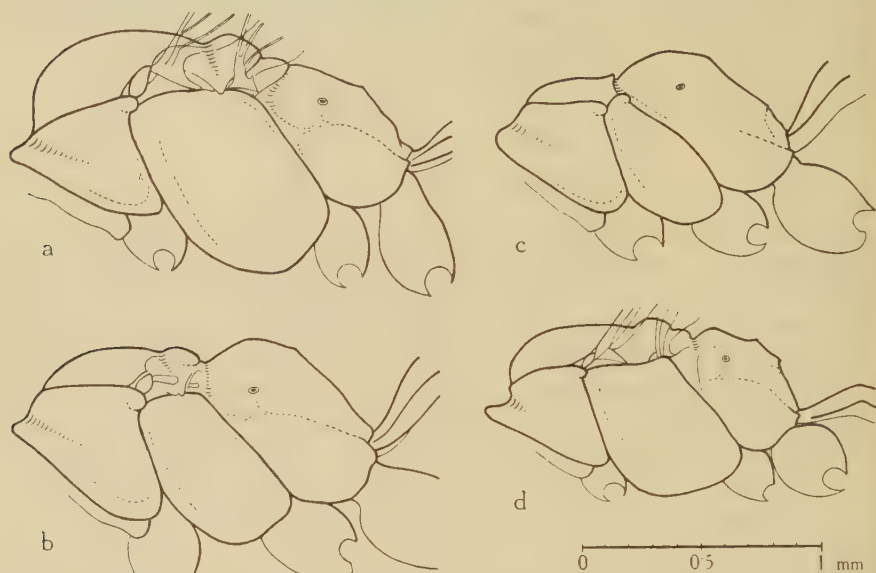


FIG. 4. Side views of the thorax and propodeum of *a*, macropterous male, *b*, micropterous male, and *c*, female of *Gelis corruptor*; *d*, winged female of *Hemiteles subzonatus*.

prominently they protrude on the vertex. In the micropterous male (fig. 5, *c* and *d*) the ocelli are so much smaller that the posterior pair are about twice their own diameter from the compound eyes and more than twice their diameter apart. They scarcely break the contours of the vertex. In the female (fig. 5, *e* and *f*) the ocelli are slightly smaller and even less protuberant than in the micropterous male. This difference is so consistent in the long series examined that every male could be classified without doubt merely by examination of its ocelli. At the same time, the development of the ocelli varies with the size of the individual in that they are comparatively the largest in the largest macropterous males and proportionately most reduced in the smallest micropterous males.

In the size of the head there is a good deal of overlap between the two forms; in fact the height and width of the heads of the largest micropterous males are nearly as great as those of the largest macropterous males. Relative to the size of the thorax, therefore, the head of the micropterous males is larger. The ratio of the width of the head to the length of the thorax in twenty-

five macropterous males was 0.54 (range 0.51 to 0.58); in twenty-five micropterous males it was 0.72 (range 0.67 to 0.80). In heads of the same width, those of the micropterous males appear to be longer and to bulge more behind the eyes, so that the occipital margin in this form appears more deeply concave than that of the macropterous males (cf. fig. 5, *a* and *c*).

The number of flagellar antennal segments ranges in micropterous males from 18 to 24 and in macropterous males from 23 to 25; and the proportions

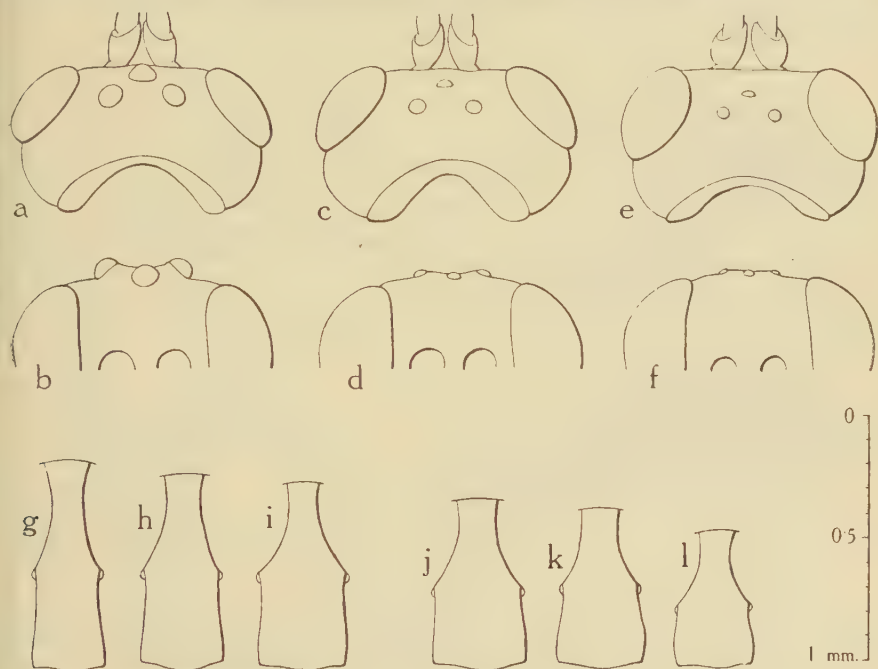


FIG. 5. *a* to *f*, dorsal and frontal views of the heads of *a* and *b*, a macropterous male; *c* and *d*, a micropterous male; *e* and *f*, a female. *g* to *l*, outlines of the petiole of six males of decreasing thoracic length, *g* to *i*, macropterous males; *j* to *l*, micropterous males.

of particular segments appear also to be correlated with the size rather than with the form of the individual.

(3) *The abdomen.* Although the difference is not completely consistent, the abdomen of the macropterous male is generally longer and narrower than that of the micropterous form. This difference is obscured by the extent to which the sclerites are retractable beneath those anterior to them, and measurements of the abdomen as a whole would therefore be misleading. The first and second post-petiolar tergites, however, are usually fully extended in dead specimens, and their length and maximum width were measured on twenty-five males of each form. The ratio of length to width was on the average 1.3 in the macropterous and 1.1 in the micropterous form, but not all of the individuals showed this difference. The abdomen of the female is in all cases relatively broader and more rounded than that of the male.

The petiole of the abdomen is also generally more slender in the macropterous form, but, as can be seen in fig. 5, *g* to *l*, the proportions of the petiole are rather variable and its shape may vary rather with the size of the individual than with its form.

(b) *Internal structures*

(1) *The muscles of flight.* A great part of the swollen thorax of the macropterous male is occupied by the indirect muscles of flight. The tergo-sternal

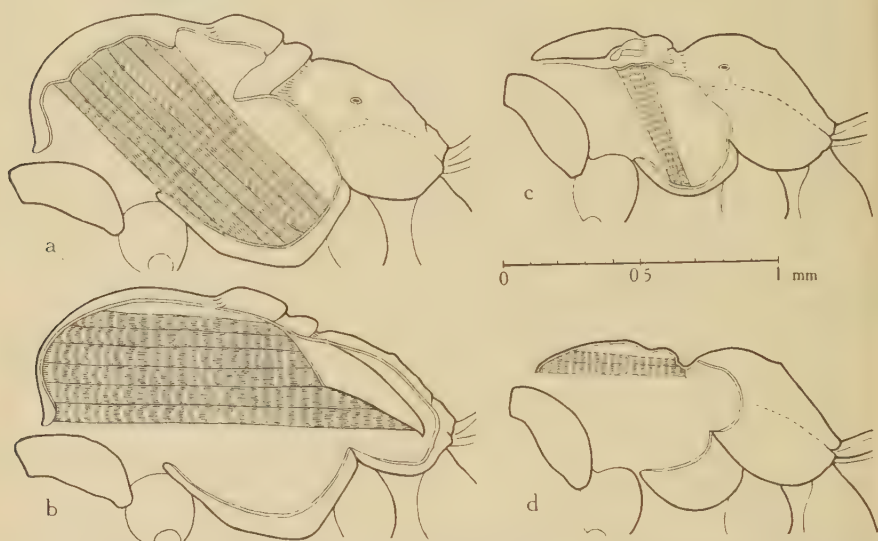


FIG. 6. The indirect muscles of flight. *a*, tergo-sternal, and *b*, longitudinal in a macropterous male; *c*, tergo-sternal, and *d*, longitudinal in a micropterous male.

muscles (fig. 6, *a*) consist on each side of twelve or fourteen large muscle bundles attached antero-dorsally to the mesoscutum and postero-ventrally to the sternum. The longitudinal muscles (fig. 6, *b*) are similarly bulky and are composed of about six muscle bundles on each side of the middle line. They are attached in front to the anterior bulge of the mesoscutum and posteriorly to the greatly elongated post-phragma, so that the longest of them are fully five-sixths as long as the whole thoracic region. The direct wing-muscles are also well developed, broad at their origins on the pleura and attached by long tendons to the axillary sclerites.

In the micropterous male the appearance of the dissected thorax is totally different. Although the indirect muscles of flight are present, they are poorly developed and occupy only a small part of the thoracic cavity. The tergo-sternal muscles (fig. 6, *c*) form a narrow band, scarcely broader than one of the muscle bundles of the macropterous male. The longitudinal muscles (fig. 6, *d*) are comparatively even more meagre, and are less than half as long as the

thoracic cavity. The direct wing-muscles are present but are not well developed. In six females dissected for the purpose, no trace could be found of either the indirect muscles of flight or the direct wing-muscles.

(2) *The post-phragma*. The indirect muscles of flight, so greatly developed in the macropterous male, are accommodated in the enlarged mesothoracic segment already described. Increase in the length of the longitudinal muscles is further provided for by the elongation of the post-phragma. In the macropterous male the shape of this structure bears some resemblance to the beak

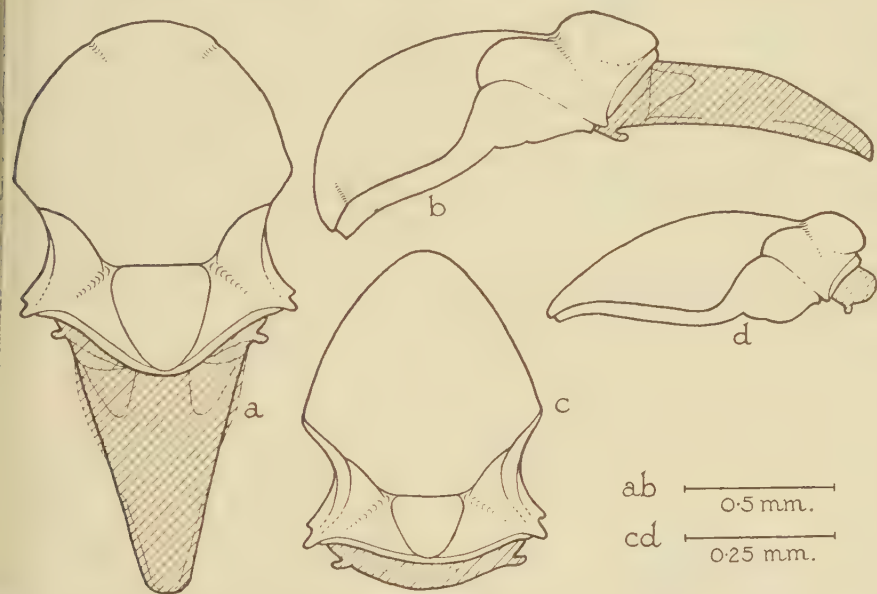


FIG. 7. The development of the post-phragma, *a* and *b*, in a macropterous male; *c* and *d*, in a micropterous male.

of a swan (fig. 7, *a* and *b*), and it is so extensively produced that it reaches the posterior part of the propodeum. In the micropterous male the post-phragma is little more than a transverse, downward-projecting ridge (fig. 7, *c* and *d*). In the female it is scarcely to be distinguished on the inner surface of the dorsum.

(3) *Other internal differences*. The differences between the three forms in the development of the muscles of flight evidently involve differences in innervation and tracheal supply. Those differences, however, are of detail rather than of gross anatomy, and their description, which cannot be brief, falls beyond the needs of this paper.

On dissection of the thoracic cavities of the three forms, the leg-muscles of the micropterous male and of the female appear to be more strongly developed than those of the macropterous male. Although they certainly occupy a greater proportion of the thoracic cavity, very detailed measurements would be necessary to show whether they are better developed in an absolute sense.

THE STRICTNESS OF THE MALE DIMORPHISM

Of the 956 males of *G. corruptor* bred in the course of this investigation, 368 are macropterous, 587 are micropterous, and one is an asymmetrical individual which must now be described. On its left side this specimen has

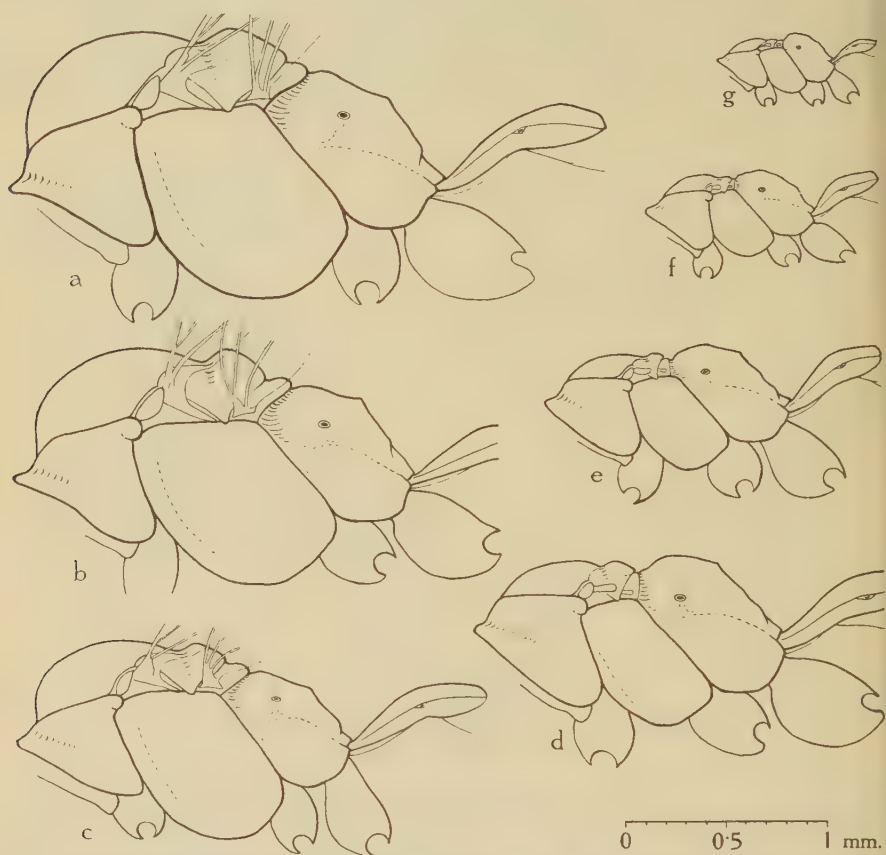


FIG. 8. Side view of the thorax and propodeum of three macropterous and four micropterous males of one strain of *Gelis corruptor*.

wing-lobes clearly of the same kind as those of a micropterous male but in each case, meso- and metathoracic, about twice as long as usual. On the right side the wings may have been fully formed but they are incompletely expanded, so that they remain only a quarter as long as fully developed wings. The thoracic length of this specimen, $16\frac{1}{2}$ units, is intermediate between that of the largest micropterous and the smallest macropterous males. Its structure is essentially that of a micropterous male: the mesoscutum is flat and does not hide the prothorax either anteriorly or laterally, the scutellum and metanotum are as in micropterous males, and the mesopleura are not swollen though they may be a little wider than usual. The ocelli are clearly asymmetrical; the

anterior and right ocelli are small as in a micropterous male, the left ocellus is large as in a macropterous male. It will be observed that this asymmetry is the reverse of that shown by the wings. The internal structures have not been examined.

While it may be of interest as showing a degree of latitude in the development of certain characters, this specimen cannot be looked upon as a normal individual representing a form intermediate between the macropterous and micropterous males. It is clearly an abnormal specimen.

Apart from this one asymmetrical individual, all the other 955 males are strictly of one form or the other, without intergradation. The wings of all are either fully developed or mere lobes; the ocelli clearly of the large or of the small type. In the specimens dissected, which are comparatively few but represent a wide range of size, the flight muscles were either fully developed or greatly reduced, and the post-phragma either elongate or a mere ridge. No intermediates between the fully developed macropterous male and the micropterous form have been observed.

TABLE 2. *The area of the prothorax, mesopleuron, and propodeum of specimens of the three forms of Gelis corruptor. (Number of squares on tenth-inch graph-paper of drawings at a linear magnification of $\times 77$)*

<i>Specimen</i>	(1) <i>prothorax</i>	(2) <i>mesopleuron</i>	(3) <i>propodeum</i>	<i>Ratio</i> $\frac{2}{1+3}$
Macropterous males				
G ⁺ ♂4. . . .	270	680	371	1.06
B7♂6. . . .	233	551	301	1.03
N1♂2. . . .	202	501	288	1.02
J8♂7. . . .	165	393	205	1.06
J8♂8. . . .	162	387	217	1.02
Micropterous males				
B7♂8. . . .	211	232	287	0.47
B7♂4. . . .	112	133	159	0.49
H ⁺ ♂17. . . .	62	64	73	0.47
K6♂10. . . .	25	30	37	0.48
Females				
456♀. . . .	149	137	212	0.38
C2♀1. . . .	134	118	182	0.37
A ♀2. . . .	94	79	123	0.36
A ♀9. . . .	62	55	81	0.38

The precision of the dimorphism of the males is quantitatively demonstrated by measurements made on the drawings for fig. 8. Side views of the thorax were drawn in order to illustrate the range of size of males within a single strain. As an afterthought, the area of the mesopleuron and of the prothorax and propodeum as seen on those drawings was measured by means of a tracing on graph paper. The series of drawings was later made up to four of each form (one additional macropterous male was drawn and measured) and the relation between the area of the mesopleuron and that of the prothorax plus propodeum

was calculated. The areas and ratios are set out in Table 2. The measurements show that over a great range of size the proportions of the thorax are very constant among individuals of the same form, and clearly different between the different forms.

This distinctness of the forms is remarkable, especially in the case of the male dimorphism determined by quantity of food where intergradation might be expected to occur.

DISCUSSION

The investigation reported above has some application to three fields of biological interest: as an example of trimorphism, as an instance of the effects of hosts on their parasites, and as a problem in morphogenesis.

Dimorphism of the female sex occurs rather frequently in the Hymenoptera; the existence of two forms of males is much less common and has been reported in only half a dozen genera. In any of these cases the species could be said to be trimorphic. The trimorphism of *G. corruptor* is especially noteworthy, however, because not only are the two forms of males markedly different but also the female is very different both from the male forms and from the form of female found in related genera.

The dimorphism of the male sex in *G. corruptor* is of special interest in four respects. In the first place the two forms are remarkably distinct and, so far as is known, are entirely without intermediates. The situation is otherwise, for instance, in the interesting case of *Melittobia chalybii* described by Schmieder (1933). There, males of the normal form have three well-developed ocelli 'while in the second-form these may be entirely absent in some individuals, or in others from one to three may be present in a more or less aborted condition' (p. 343); and other differences, the size of the abdomen and wings and the colour of the body, are not very clear-cut. In respect of its strictness, the dimorphism of *G. corruptor* appears to differ from most of the cases that have been reported in male Hymenoptera.

Secondly, the differences between the two forms are both striking and deep-seated. They involve not only the wings but also the gross structure of the trunk, endo-skeletal parts, muscles, sense organs, and the nervous and respiratory systems. The extent of this dimorphism is greater than has been described in other cases. It is likely, however, that in some of those cases, where two forms have been said to be identical apart from the presence or absence of wings, a more careful study will reveal additional differences, at least in the muscles and endo-skeleton.

In the third place, it is noteworthy that the differences between the two male forms of *G. corruptor* all appear to be bound up with the loss of the power of flight. The almost complete reduction of the wings in the micropterous male makes powerful flight muscles unnecessary, the post-phragma need not be extended for their attachment, the mesothorax need not be capacious to contain them, the abdomen can be shorter and broader since its elongation is not required for stability in the air. Even the difference in the ocelli may

be part of this complex of structural changes, since the ocelli are known in several insects to be stimulatory organs and in some cases their activity may be necessary for flight. These inter-connected alterations observed in the micropterous male are carried even further in the apterous female, where the wings and flight muscles are completely lost, the thoracic structure is greatly simplified, the post-phragma and ocelli are small, and the abdomen is short and very broad.

Fourthly, the dimorphism is of interest as providing a third case in the Hymenoptera in which the appearance of one form or the other has been brought under experimental control. Eggs and young larvae of the hive bee can be interchanged between queen and worker cells to produce the female or worker caste. A laying adult of *Trichogramma semblidis* can be led to oviposit in hosts that will give one or the other of its two male forms. Larvae of *G. corruptor* can be fed on a large host to produce the macropterous form or starved to produce the micropterous. The last case allows a more rigorous experimental control than either of the others. Indeed, in all four respects outlined above, the case of *G. corruptor* claims particular attention as providing valuable material for the demonstration and study of dimorphism.

Observations made in the course of this investigation contribute to insect parasitology by providing another instance of the effect of different hosts on a parasite. A number of cases have accumulated which show that in a variety of ways a host may exert a profound effect on the individual parasitoid that destroys it. The emergence of two distinct forms of *G. corruptor* from *Nemeritis* and *Apanteles* is another example of the phenomenon, and one in which the morphological effects are more striking than in any case known except perhaps that of *Trichogramma semblidis*. Nevertheless, the case of *G. corruptor* is of less theoretical interest than that of the *Trichogramma* because in this instance it is the quantity of food provided by the host, rather than something specific to it, that produces the effect.

It was neither to describe another case of dimorphism nor to exemplify again the effects of a host on its parasite that this investigation was begun. The object of the work was to find material suitable for an attempt on another problem—that of the morphogenetic processes involved in the kind of dimorphism exhibited by males of *T. semblidis*. In some respects, at least, the search has been successful. We have, in the young male larva of *G. corruptor*, an individual which is capable of developing into either the macropterous or the micropterous form. In the course of its development, its final structure is determined so that ultimately it conforms to one of two distinct and markedly different patterns. The essential morphogenetic problem lies in the nature of that determination, in the question what factors lead each individual male along either one of two different and sharply divergent lines of development.

The study of *G. corruptor* described in this paper already carries the investigation two steps beyond the point that was reached in the work on *Trichogramma semblidis*. It has been possible to show that the two male forms are not based on a different genetic constitution, so that each egg has the

potentialities of both forms of male. It has also been shown, by experimental transfer of eggs and young larvae, that the determination takes place during development between the early larval stage and the pupa. Whether the material can be made to disclose precisely what factors determine the phenotype and what morphogenetic processes take place as the determination is effected remains to be seen.

ACKNOWLEDGEMENTS

I am indebted to Rev. E. J. Pearce, C.R., for collecting the *Gelis* from which most of my strains were established; and to Mr. J. F. Perkins, of the British Museum, for identifying many specimens in this difficult genus.

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Enucleation of Sea-Urchin Blastomeres with or without Removal of Asters

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SUMMARY

The following experiments were carried out on one blastomere at the two-cell stage of the sea-urchin egg:

1. Removal of the nucleus.
2. Removal of the nucleus and surrounding cytoplasm (centrosphere).
3. Attempted removal of the centrosphere only.

In cells from which the nucleus had been removed the asters multiplied for up to 7 hours and then disappeared. During the period of aster multiplication, cleavage furrows often appeared between the asters but did not succeed in dividing the cell. After about 8 hours, cleavage into a large number of spheroidal cells occurred.

When the nucleus and centrosphere were removed no asters were formed and no cleavage furrows were observed until after about 8 hours, when division into a large number of spheroidal cells occurred.

When attempts were made to remove the centrosphere, leaving the nucleus intact, asters were always eventually re-formed. Before the re-formation of asters neither the nucleus nor the cytoplasm divided although dissolution of the nuclear membrane, followed by its reappearance, occurred. After re-formation of the asters cleavage appeared to be normal.

INTRODUCTION

WHILE experiments were being carried out on differentiation in the eggs of sea-urchins, it was noted that some of the enucleated blastomeres developed asters while others did not. But all the enucleated cells eventually divided, whether asters had been initially present or not (Lorch *et al.*, in press). This phenomenon deserved closer investigation. The question also arose whether cleavage would occur if the nucleus was left in the cell but the asters removed.

In view of the confusion which exists regarding the nomenclature of cytoplasmic structures, I shall define the terms used in this paper.

Microdissection studies carried out by Chambers (1917, 1919, 1951) showed that the cell aster is a reversible gelation phenomenon. This is confirmed by Marsland's (1951) experiments in which dividing eggs were subjected to hydrostatic pressures. In cells fixed immediately after exposure to pressures of 5,000–6,000 lb. per square inch, no trace of spindle or asters was found. But if several minutes were allowed for recovery, the asters appeared again.

The *monaster* is a gelated body with numerous radiations about a hyaline central zone. It is normally formed after sperm entry (*sperm aster*) but also

occurs after artificial parthenogenesis (Chambers, 1921), even in the absence of the female nucleus (Harvey, 1940a), in which case it is called a *cytaster*. The hyaline central region of the monaster has been variously called the centrosome, astrosphere, or centrosphere. I shall use the last term. The *centrosphere* is a lake of fluid enclosed by the gelated material of the aster. Several observers have stated that granules move from the periphery of the centrosphere towards the outer end of the astral rays. This has been interpreted by Chambers to mean that the radiations of the aster are delicate paths of centripetally streaming fluid.

Inside the centrosphere is said to be a centriole which is regarded as a permanent cell organ, the division of which initiates the division of the whole astral system, followed by cleavage of the cell (Wilson, 1925, p. 672). However, centrioles are not visible in living sea-urchin eggs and it has been shown, at least for the species *Echinarachnius*, that they are fixation artifacts (Fry, 1929a, b; Fry *et al.*, 1929).

The *amphiaster* is often referred to as the 'achromatic figure'. It consists of two asters (centrospheres+astral rays) with a spindle between them (Gray, 1931, p. 155). The formation of the amphiaster in *Echinarachnius* eggs has been described by Chambers (1917). A similar process takes place in the eggs of *Paracentrotus lividus* and of *Psammechinus miliaris*, which were used for the present experiments.

The interphase nucleus is surrounded by a clear, nongranular area from which the centrospheres arise. Astral rays appear in the granular cytoplasm and grow until they extend throughout the cell. Simultaneously the nucleus becomes bipyramidal and then ovoid just before dissolution of the nuclear membrane. After the disappearance of the nuclear membrane the spindle can be seen lying between the asters (fig. 2, A, normal half). Then the asters get fainter at the periphery and the cleavage furrows appear. At this stage no trace of the nuclear components can be seen without the use of special microscopic methods until the nuclei are reformed in the daughter cells. The asters gradually fade, leaving round each nucleus a granule-free zone which gives rise to a new set of centrospheres (fig. 1, A).

Three types of experiments will be described: (1) removal of the nucleus without the centrospheres, (2) removal of the nucleus *and* the centrospheres, (3) attempted removal of the centrospheres only.

METHOD AND MATERIAL

Most of the experiments were performed on eggs of *Paracentrotus lividus*—some enucleations were also carried out on eggs of *Psammechinus miliaris*. All operations were performed on eggs from which the fertilization membrane had been removed within 2 minutes of fertilization. The eggs were placed in hanging drops of sea-water in a paraffin chamber (Commandon and de Fonbrune, 1938) and allowed to complete the first cleavage. The nucleus of one of the two blastomeres was then removed by means of a micropipette, the diameter of which was somewhat smaller than that of the nucleus. The egg was held

lightly in a glass microhook. The de Fonbrune micromanipulator was used. If it was desired to remove only the nucleus, care was taken to withdraw the pipette as soon as the nucleus had entered the tip. If it was intended to remove also the centrospheres the clear cytoplasm surrounding the nucleus was sucked out. (It has previously been shown that up to 50 per cent. of the cytoplasm of an egg can be sucked out without adverse effect on development (Hörstadius *et al.*, 1950). Removal of the cytoplasm which gives rise to the asters proved to be more difficult when it was desired to leave the nucleus behind. Suction was applied with a very fine pipette as close as possible to the nucleus, yet without touching the latter. In this way most or all of the clear sphere round the nucleus (future centrospheres) can be removed.

RESULTS

1. Removal of the nucleus only

Immediately after the nucleus had been sucked out a light area was seen at the centre of the blastomere. This zone enlarged and often persisted for some time (fig. 1, A). It then gave rise to two centrospheres from which astral rays radiated and extended throughout the cell, exactly as in the normal cell (fig. 2, A). These asters may form simultaneously in both cells or, more commonly, there was a delay in the operated cell. The spindle is clearly seen in normal blastomeres as a refractile biconvex body lying between the centrospheres. In the enucleated blastomeres a light streak was sometimes seen between the asters, especially in the *Psammechinus* eggs, so that only close examination revealed a difference between the normal and enucleated blastomeres (fig. 2, A). This connecting streak disappeared, leaving two distinct asters (fig. 2, B). In most cases these two asters divided, giving rise to 4, 8, 16, &c., distinct asters. This process of division of the asters was usually correlated with cell cleavage in the normal half of the egg, but where there was an initial delay in the enucleated cell the number of asters did not correspond to the number of cells in the intact half (fig. 1, A-C).

The asters continued to multiply in the enucleated cell for up to 7 hours, after which the rays faded and finally only faint white areas (vestiges of the centrospheres) were seen. During the period of aster multiplication, division furrows were often formed between two asters, but these furrows disappeared again and never resulted in cleavage (fig. 1, B and C). This suggests that the furrows, although they sometimes *appear* to divide the cell, are not complete; a last link remains between the cells, which subsequently fuse again. The furrows tended to appear and disappear several times. After the asters had finally faded away a different process of division started which may be termed pathological cleavage: the cell rapidly (i.e. in 10-30 minutes) divided into many small spheroid cells of irregular sizes (fig. 1, D). This usually happened between 7 and 8 hours after enucleation, at a time when the normal half was beginning to form a blastula which grew over the enucleated half. The mass of non-nucleate spheroidal cells became enclosed in the cavity of the blastula and remained there until the pluteus stage which is as far as the larvae were

observed. The fate of these non-nucleate cells is considered in another paper (Lorch *et al.*, in press). The significant facts here are (a) that aster-formation and multiplication proceeded in the enucleated blastomere fairly normally but

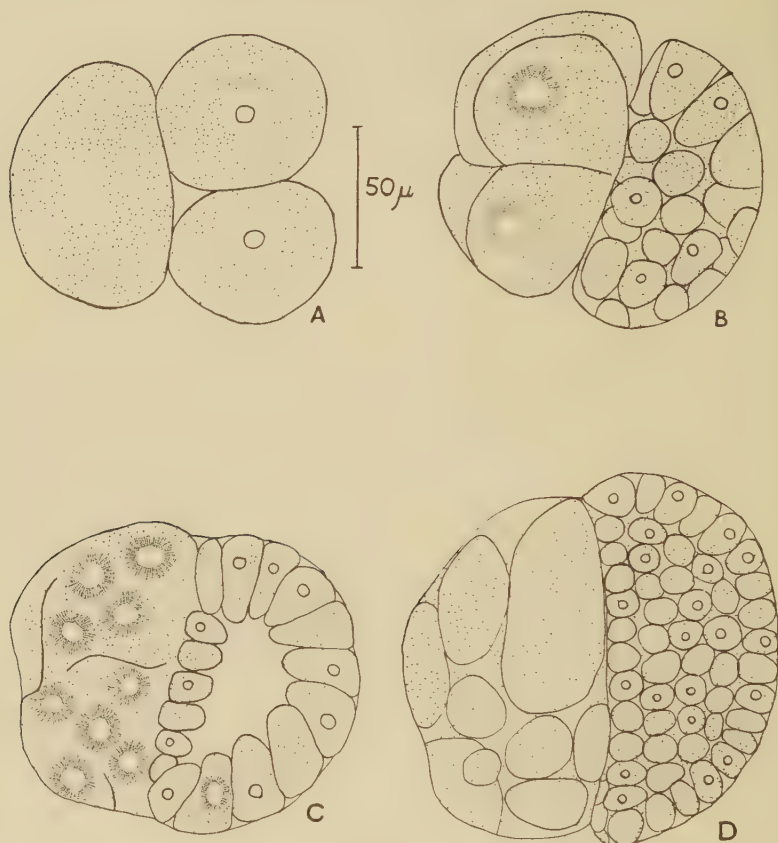


FIG. 1, A-D. Successive stages in the development of a *Paracentrotus* egg. The nucleus, but not the centrosphere, of one blastomere (left side) was removed at the 2-cell stage. (This figure, like figs. 2, 3, and 5, is a camera lucida drawing of living cleavage stages.) A, 55 minutes after enucleation. B, 3 hours after enucleation. 4 asters present (only 2 shown in surface view). Note apparently normal 'cleavage' furrows. C, 6 $\frac{1}{4}$ hours after enucleation. Many asters are present in the enucleated cell. The 'cleavage' furrows have receded. D, 8 $\frac{1}{4}$ hours after enucleation. The enucleated half has divided irregularly. No asters are now seen.

no permanent division furrows were formed, (b) after disappearance of all visible organization from the cytoplasm rapid cleavage into a great number of small units took place. These may fairly be called cells. The semipermeability of the membranes of these cells remains intact for some days, as can be demonstrated by adding trypan blue to the water. The non-nucleate cells remain unstained, whereas dead (cytolyzed) material rapidly becomes blue. Moreover, these cells have the capacity to divide into still smaller units.

Removal of the nucleus and centrospheres

This resulted in a cell devoid of any visible structures (fig. 3, A). The cell remained unchanged for 6–8 hours, by which time the normal blastomere had formed a blastula. About this time the enucleated half underwent pathological

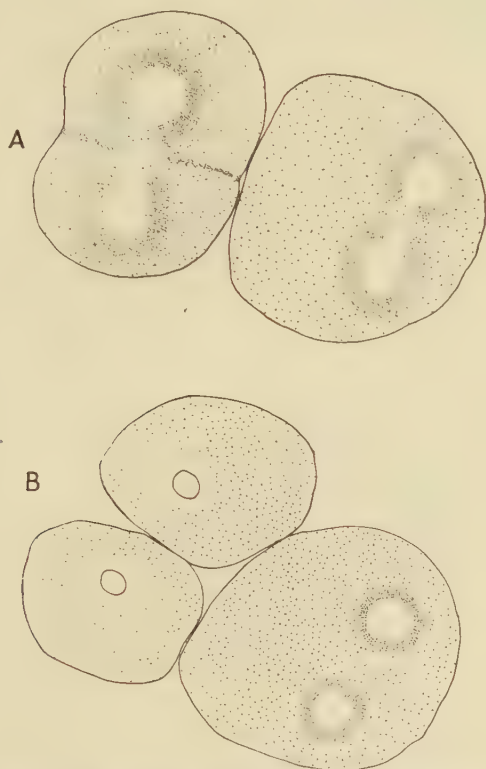


FIG. 2. Cleavage stages of *Psammechinus miliaris*. The nucleus, but not the centrosphere, of 1 blastomere (right side) was removed at the 2-cell stage. A, 25 minutes after enucleation. Note the almost normal appearance of the 'amphiaster' in the operated cell. B, 35 minutes after enucleation: 2 separate asters are seen in the operated cell.

cleavage as described above, i.e. a mass of small irregular cells was formed (fig. 3, B and C). The early, incomplete, temporary division furrows noticed in cells with asters were absent here. They thus appear to be connected with aster-formation, whereas the later cleavage into many cells takes place irrespective of the initial presence of asters.

Attempted removal of the centrospheres only

The results of enucleation described under 1 and 2 are based on a large number of experiments.

Removal of centrospheres, however, succeeded in only four cases and this

must be considered as a preliminary experiment. Any conclusions based on it may have to be modified later.

The experiments were performed on eggs of *Paracentrotus lividus*. In all cases the centrospheres were sucked out of one blastomere at the two-cell stage. The attempt was made to remove all the clear area round the nucleus as well as the astral rays which had begun to form in the surrounding cytoplasm.

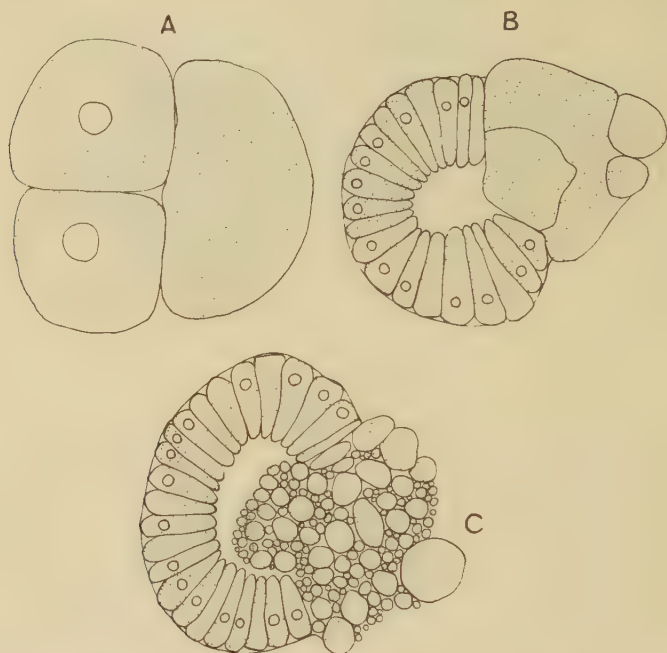


FIG. 3. Cleavage stages of *Paracentrotus lividus*. The nucleus and centrosphere of 1 cell (right side) were removed at the 2-cell stage. A, 35 minutes after enucleation. The operated cell is uniformly granular and remained unchanged for 7 hours. B, 7 hours after enucleation. Irregular division of the operated cell is beginning. C, 7½ hours after enucleation. The operated half has divided into many spheroidal cells.

In doing this it was noticed that within a few minutes of sucking out the astral rays they had begun to re-form. So the suction was continued until no more rays appeared. In the successful experiments the nucleus remained distinct and unchanged, but in many cases the nucleus entered mitosis in spite of the continual manipulation of the surrounding cytoplasm. The nucleus seemed to be attached to the centrospheres and could be pulled about the cell by applying suction to the clear centrospheres, but not by sucking out the granular cytoplasm.

It has previously been mentioned that just before dissolution of the nuclear membrane the nucleus becomes ovoid—as though it were stretched between the centrospheres. If, at this stage, the clear cytoplasm of the centrospheres was sucked out, the nucleus returned to its spherical form (fig. 4).

In two experiments where the centrospheres and all visible astral rays had

been removed, a monaster, followed by the amphiaster, was re-formed after 40 and 60 minutes respectively. Nuclear division followed by cytoplasmic cleavage thus took place 50 and 75 minutes after the operation. This represents a delay of 33 and 58 minutes as compared with the time between the 1st and 2nd divisions of normal blastomeres. The operated cells then continued to divide normally, although they were 'out of step' with the normal half. Three hours later one of the operated blastomeres had formed 16 cells, the other 4 cells. The normal blastomeres of both eggs had by this time reached an early blastula stage.

In the third experiment the nuclear membrane disappeared although the centrospheres and astral rays had apparently all been sucked out. In the place



FIG. 4. Diagram illustrating shape of nucleus before and after sucking out the centrospheres.

of the nucleus a light area was seen. No astral rays re-formed. This state lasted for 70 minutes. Then the nuclear membrane reappeared and an apparently normal nucleus was seen, surrounded by a clear area (the new centrosphere). Twenty minutes later a normal amphiaster was seen and cleavage took place 102 minutes after the operation. This represents a delay of 84 minutes as compared with the normal cell. The latter had given rise to 8 cells at the time the operated cell entered division. Six hours after the operation the blastula stage was reached and the unequal sizes of the cells was no longer apparent.

The course of the fourth experiment resembles the one just described, i.e. the nuclear membrane dissolved and re-formed. But the delay at all stages was even greater. Twenty minutes after sucking out the centrospheres and astral rays the appearance of the cell was as shown in fig. 5, A. The nucleus is clearly seen. Some faint radiations have re-formed in the cytoplasm.

Thirty-five minutes after the operation a clear area appeared on one side of the nucleus (fig. 5, B), and this gave rise to a monaster. The nuclear membrane disappeared 45 minutes after the operation (fig. 5, C). The monaster reached its maximum development simultaneously with the amphiaster in the normal half which at this time entered the '8'-cell stage (i.e. 2 cells dividing into 4). Sixty-five minutes after the operation the monaster faded away, leaving a clear space in which some small bodies (? chromosomes) were seen. At this stage the four normal cells were in interphase (fig. 5, D). Forty minutes later (i.e. 105 minutes after the operation) the normal cells had divided into 8 cells ('16'-cell stage) but the operated cell was unchanged. After a further 20 minutes the

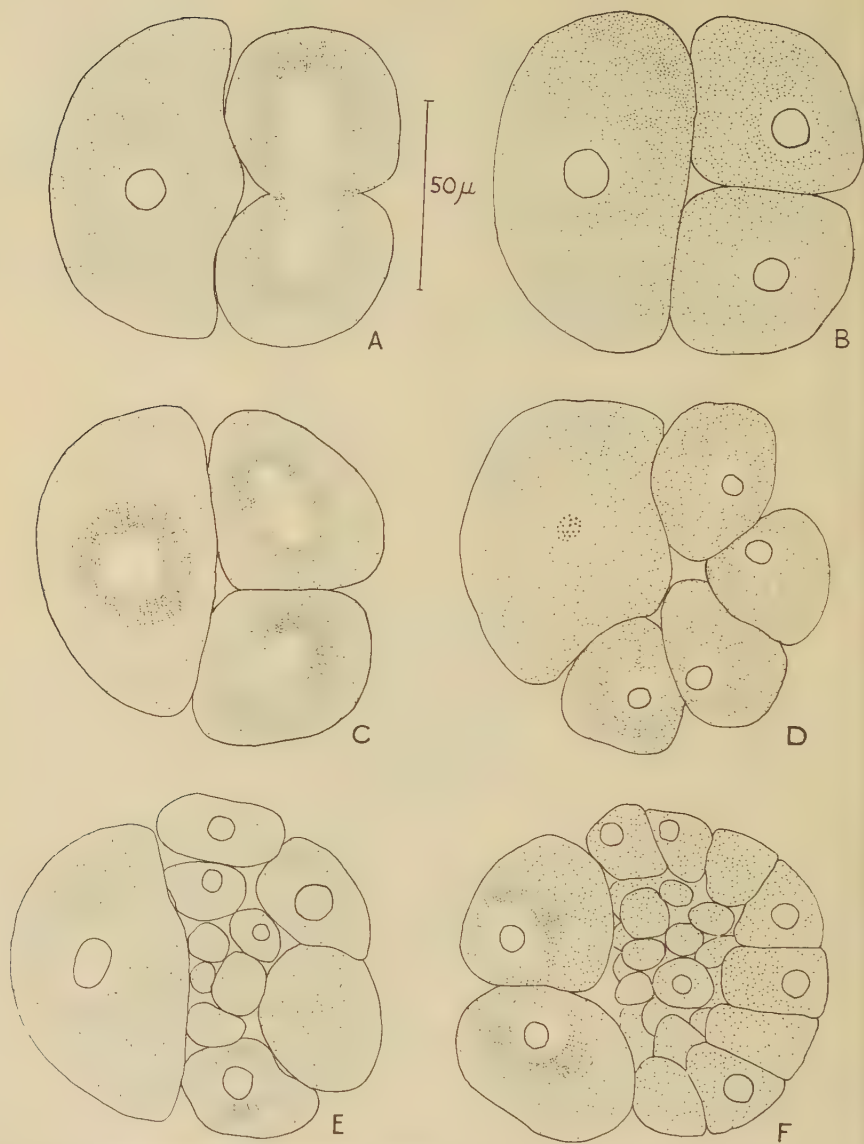


FIG. 5, A-F. *Paracentrotus lividus*. Attempted removal of centrospheres of 1 cell at the 2-cell stage. Operated cell on the left. A, 20 minutes after operation. Some faint radiations have reappeared in the cytoplasm. B, 35 minutes after the operation. Nucleus intact. C, 45 minutes after operation. A large monaster has formed. D, 65 minutes after operation. The astral rays have faded. Chromosomes seen in the centrosphere. E, 2 hours 45 minutes after operation. The nuclear membrane has re-formed. An apparently normal amphiaser is about to form. F, 3 hours 15 minutes after operation. The operated cell has divided.

surface of the operated cell became irregular and pseudopod-like protrusions appeared. This state continued for 30 minutes and then the surface became smooth again and the nucleus was re-formed. The shape of the nucleus rapidly changed from spherical to ovoid as centrospheres and astral rays began to appear 165 minutes after the operation (fig. 5, E). The normal half was at the 32'-cell stage (16 cells).

Five minutes later the nucleus of the operated cell disappeared and a normal amphiaser was seen. After a further 25 minutes the second cleavage was completed. Thus the time between the first and second cleavage of the operated cell was 195 minutes as compared with 17 minutes in the normal blastomere.

Six hours after the operation the normal half had formed an early blastula while the operated half was just entering the '16'-cell stage (8 cells).

To sum up: in two of the four cases where asters were sucked out of one blastomere at the 2-cell stage, nuclear division, followed by cytoplasmic cleavage, took place after 50 and 75 minutes respectively. During this time the asters were regenerated.

In the other two cases the removal of the astral material seems to have been more complete. The nuclear membrane dissolved, indicating that the nucleus had entered prophase. But after a certain delay a *single* nucleus was re-formed. Cytoplasmic cleavage only followed a *second* dissolution of the nuclear membrane, accompanied by re-formation of the amphiaser. The times between the operation and cleavage were 102 and 195 minutes respectively.

DISCUSSION

Since Boveri's (1896) statement that permanent cell division only takes place between two asters *connected by a spindle*, although temporary and incomplete furrows may be formed between isolated asters, the question of whether non-nucleate cells can divide has been much debated. There is abundant evidence, derived from a variety of species, that the egg cytoplasm is capable of producing asters and also of dividing in the absence of nuclear material. Ziegler (1898) observed one case of independent cytoplasmic division in an egg of *Echinus microtuberculatus* in which the egg and sperm nuclei came to lie in one blastomere after the first cleavage. Thus the other blastomere had no nucleus. Yet asters appeared and multiplied in this non-nucleate cell and irregular cleavage furrows were formed. Finally, the cell divided into a great number of spheroidal cells. At this time the nucleated half had produced a blastula. The appearance of this embryo in Ziegler's drawings is strikingly similar to the corresponding cases in the present series, i.e. where the nucleus of one blastomere was removed, leaving the centrosphere behind (fig. 1). Wilson (1904) observed cleavage in non-nucleate fragments of mollusc eggs and noted that these fragments pass through alternating periods of activity and quiescence corresponding to the division rhythm of the nucleated portion.

A similar phenomenon has been observed in the present experiments where aster formation in the enucleated blastomere often—though not invariably—kept in step with the corresponding process in the nucleated cells.

It may be noted here that since all experiments were carried out at the 2-cell stage, the unoperated blastomere serves as a constant control for the operated cell with which it is in contact. Thus changes in the nucleus and cytoplasm and division rates can be directly compared.

Aster formation in non-nucleate cells or cell fragments has been described by many workers. Such 'cytasters' were first noted by Morgan (1896) in whole *Sphaerechinus* eggs treated with hypertonic sea water. Yatsu (1905) treated non-nucleate fragments of sea-urchin eggs with CaCl_2 in sea-water and asters were invariably formed.

McClendon (1908) induced parthenogenesis in *Asterias* eggs after sucking out the polar spindle. These non-nucleate eggs formed numerous asters and incomplete division furrows. The eggs then divided simultaneously into several cells of different sizes, each cell containing one or more asters. Such irregular cleavage of non-nucleate eggs or egg fragments was also described by Jollos and Peterfi (1923) in the axolotl, by Fankhauser (1929, 1934) in *Triton*, and by Harvey (1936, 1940b) in *Arbacia*. Fankhauser (1934) describes two types of cleavage of the non-nucleate fragments according to whether asters are present or not. In the first case division is delayed and incomplete but still fairly regular. If, however, no asters are present an 'irregular fragmented mass' is produced by simultaneous cleavage which suggests a 'breaking up of cytoplasm on the verge of cytolysis'. It has been shown that enucleated sea-urchin blastomeres also show these two types of cleavage to some extent: first the incomplete and reversible furrows which appear in the presence of asters and later the rapid division into a mass of cells which occurs in the absence of asters. But the latter process—which I have called 'pathological cleavage'—can hardly be called a breaking up of the cytoplasm since the resulting cells remain intact for days.

Thus the facts which emerge from a study of the literature and the present work are that non-nucleate cells or cell fragments, whether in contact with their nucleated counterpart or not, are capable of producing asters. The asters multiply for a certain period but eventually fade. During this time division furrows may or may not appear. Cytoplasmic division is certainly not an invariable concomitant of aster formation. Conversely asters are not essential for cytoplasmic division. Differentiation of non-nucleate cells has never been shown to occur. This question is discussed in another paper (Lorch *et al.*, in press).

An observation regarding the spacing of the asters in the non-nucleate cells may be of interest here. The asters appear to be distributed at random throughout the cell, i.e. they do not reflect the cleavage pattern of the sea-urchin egg which, at the 16-cell stage, displays a typical arrangement of 4 micromeres, 4 macromeres, and 8 mesomeres. In another connexion (Hörstadius *et al.*, in press) observations were made on cells enucleated at the 8-cell stage, not as in the present series at the 2-cell stage. If the centrosphere was left intact these cells formed asters almost immediately after enucleation, and here the configuration of the asters varied according to the type of cell operated: in vegetal cells the plane of the asters was nearly parallel to the egg axis, whereas in

animal cells it was parallel to the equatorial plane. This is exactly what happens in normal vegetal and animal cells before the formation of micro- and macromeres, or mesomeres. Thus, although the enucleated cells do not divide at this stage they reflect the cleavage pattern of the normal cell. Similarly, enucleated micromeres (16-cell stage), whether isolated or *in situ*, show the typical asters of a cell about to divide into submicromeres.

With regard to the possibility of nuclear division in the absence of asters we cannot come to any definite conclusion owing to the experimental difficulties of removing every trace of 'aster precursor'. In two of the present experiments the nucleus entered prophase, but in the absence of the amphiaster, the nuclear membrane was reconstituted. This course of events is paralleled in the experiments described by Wilson (1925, p. 168), where the monaster formation in sea-urchin eggs was induced by shaking or by various chemicals. Here, too, the nucleus entered mitosis, but a single tetraploid nucleus was formed. This process repeated itself several times until finally an amphiaster was formed and nuclear division followed. Polyploid larvae resulted. It seems that the separation of the chromosomes which normally takes place at anaphase is not possible in the absence of the amphiaster. It has been shown that the asters are regenerated in the sea-urchin blastomere so long as *either* the nucleus *or* the centrosphere is intact, whereas blastomeres deprived of both nucleus and centrospheres never form asters. It is remarkable that, whereas it is easy to remove the nucleus and leave aster-forming material behind, it is almost impossible to remove all the aster-forming cytoplasm while leaving the nucleus intact. It seems to me that Fry *et al.* (1929) have conclusively shown that the 'centriole' in sea-urchin eggs is an artifact. Yet textbooks of cytology (Gray, 1931; Gresson, 1948) still state that the centrosphere contains one or two granules, called centrioles, which are self-perpetuating division centres and give rise to the asters which then initiate cleavage. If such a centriole were present in sea-urchin cells (it is said to lie very close to the surface of the nucleus—Gray, 1931, p. 157) then removal of the nucleus would result in removal of the centriole. Hence no asters should be formed in non-nucleate cells. If the centriole were in the cytoplasm, at some distance from the nucleus, it might be left behind when the nucleus is sucked out and thus initiate asters. But then it becomes hard to explain how asters are regenerated after persistent sucking out of the cytoplasm surrounding the nucleus.

From the present experiments it appears more likely that any part of the cytoplasm is capable of participating in the formation not only of scattered 'cytasters' which are not necessarily division centres, but also of the normal amphiaster without which mitosis does not go to completion.

This work was carried out at the Station Biologique, Roscoff, France, during the summer of 1951. I should like to thank Professor Theissier, Professor Drach, Dr. Bocquet, and Dr. Lévi for their unfailing assistance in obtaining the necessary material. A part of the expenses was defrayed by a grant from the Royal Society, for which I should like to record my thanks.

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Some Structural Proteins of *Mytilus edulis*

By C. H. BROWN

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With one plate (fig. 7)

SUMMARY

1. The byssus threads, periostracum, hinge, ground substance of the shell, and the supporting material of the gills of *Mytilus edulis*, have been examined by physical, chemical, and histochemical means.
2. The byssus threads, periostracum, and hinge have been shown to consist of a quinone-tanned protein.
3. The byssus threads are formed in the posterior groove of the foot from the secretions of two glands, the 'white' gland which supplies the bulk of the protein of the thread, and the 'purple' gland which supplies the aromatic material responsible for the tanning. The periostracum is secreted by gland cells in the epithelium of the outer lobe of the mantle edge.
4. The supporting material of the gills is a fibrous protein without quinone-tanning. This protein is in some respects similar to the untanned protein of the byssus.

INTRODUCTION

IN *Mytilus edulis*, structural proteins—that is, proteins concerned with support or protection rather than with the movement or metabolism of the animal—occur in the byssus, periostracum, and ground substance of the shell, in the hinge between the two valves of the shell, and in the supporting material of the gills.

METHODS

These structures have been investigated by methods described in a previous paper (Brown, 1950), which enable one to determine the types of linkage between protein molecules in any particular structure; on these depend its mechanical and chemical properties. Polarized light and X-ray diffraction data have been used to determine the arrangement of the protein molecules, and the morphology and mode of formation of these structures have been examined by standard histological and histochemical techniques.

RESULTS

The byssus

A review of early papers dealing with the byssus of lamellibranchs is given by Boutan (1895). One of the earliest is that of Antoine von Heide (1684), who believed that the byssus was a sort of plant with its roots in the foot of the animal, but it was Réaumur (1711) who gave the first accurate description of [Quarterly Journal of Microscopical Science, Vol. 93, part 4, pp. 487–502, Dec. 1952.]

the byssus of *Mytilus* itself. He distinguished the two main visible parts: the trunk or stem and the fine threads attached to the stem, and he recorded observations on the growth and regeneration of the byssus and noted the part played in its formation by the posterior groove of the foot.

Müller (1837), Tullberg (1877), Carrière (1879), Barrois (1885), Jobert (1882), Coupin (1892), Boutan (1895), and Frierson (1903) investigated the byssus in a wide range of lamellibranchs, and all came to the conclusion that the byssus is an attachment organ secreted by special glands of the foot. Both

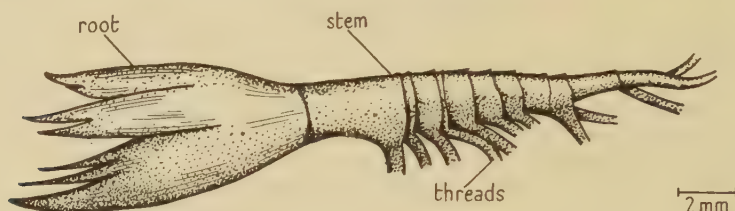


FIG. 1. *Mytilus* byssus, with threads cut short.

Müller and Tullberg gave full descriptions of the byssus and byssus-gland in *Mytilus*. Their accounts were later amplified by Williamson (1907). In one respect only has the present study made necessary a modification of Williamson's account (see p. 490).

Three parts are distinguishable in the byssus (fig. 1).

- (i) The root, embedded in the byssus-gland, which opens posteriorly at the junction of the foot with the visceral mass.
- (ii) The stem, continuous with the root but not embedded in the byssus-gland.
- (iii) The threads attached to the stem.

In the individual threads four distinct regions can be recognized (fig. 2).

- (i) At the distal end is an adhesive disk by which the thread is attached to the substratum.
- (ii) The disk is joined to a cylindrical, stiff, smooth portion which amounts to approximately two-thirds of the length of the thread.
- (iii) The remaining third of the thread is flattened and less stiff, and its surface is corrugated.
- (iv) The thread ends proximally in a ring of material by which it is attached to the stem.

Though seemingly composed of a number of separate parts, the byssus is, in fact, a continuous structure. The root itself is made up of thin, fibrous sheets which lie in the antero-posteriorly laminated byssus-gland in which they originate. On passing out of the byssus-gland, the fibrous sheets are compressed and folded by the mouth of the gland, and the cylinder of folded laminae so formed becomes coated with material secreted at the mouth of the

gland. This cylinder of folded laminae forms the core of the stem (figs. 3 and 4). It increases in length and diameter as more and more material emerges from the byssus-gland, forced out by secretion-pressure in the gland, so that

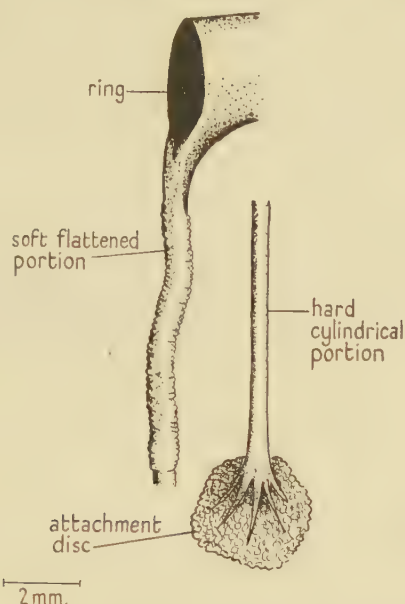


FIG. 2. Byssus-thread; one third of the hard, cylindrical portion is omitted.

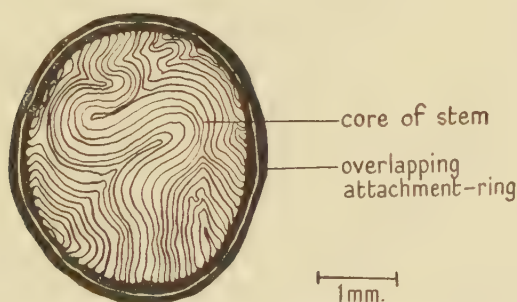


FIG. 3. Transverse section of byssus-stem to show fusion of the root-laminae with the material secreted by the lips of the byssus-gland to form the attachment-rings of the threads.

the distal end of the core is the oldest part of the byssus. The threads themselves are secreted in the groove that runs down the posterior surface of the foot and ends in a sucker-like depression, where the attachment disk is formed. At the junction of the thread with the stem, the thread fuses with a ring of material secreted by the mouth of the byssus-gland. This ring in turn is fused along its proximal edge with the fibrous laminae of the root. As the fibrous laminae of the root increase in length, the stem lengthens and the threads

are carried farther and farther away from the opening of the byssus-gland and from the body. The distal edge of each attachment-ring is free, that is, not fused to the stem, and overlaps the fused portion of the next more distal ring (fig. 4). This suggests that the formation of the threads is associated with periodic secretory activity of the mouth of the byssus-gland, and that the newly secreted material will not fuse with that last secreted. The threads may be produced so rapidly that their attachment-rings come to overlap each other. As more and more threads are added, the stem grows in thickness.

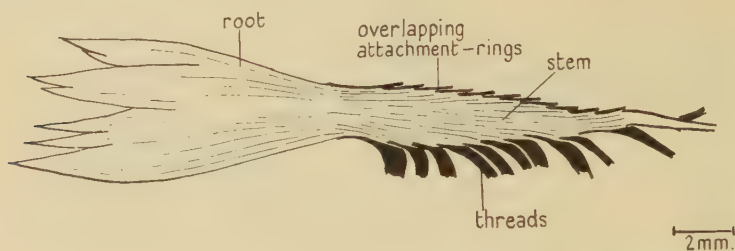


FIG. 4. Longitudinal section of the byssus to show the arrangement of the attachment-rings of the threads and their relation to the fibres of the root-laminae.

Williamson's description differs only from the above in that he failed to observe the fusion of the attachment-rings with the fibres of the root-laminae.

Physical properties of the byssus

The byssus-threads are translucent and yellowish-brown when young, turning dark purple or purplish-brown with age. This colour-change is particularly noticeable in the attachment-disk. The stem has the same appearance as the threads, though it rarely exhibits the marked purple colour found in the attachment-disks. The root is white and opaque.

The entire threads are tough and extensible, and it is possible to stretch them to nearly twice their initial length without breaking them; on release they return almost completely to their initial length. Most of this extension takes place in the corrugated upper portion of the thread, however. In such a stretch this must itself be extended by some 400 per cent. The rubber-like low elastic modulus and reversible extensibility exhibited by the byssus threads are characteristic of feebly cross-linked systems of long chain molecules (see Picken, 1940).

Between crossed nicols, all parts of the byssus show well-marked birefringence, positive with respect to the length of the threads and to the length of the fibres of the root laminae. Transverse sections of the threads are completely isotropic, showing that the fibres in the laminae are extremely well oriented, and that the birefringence is uniaxial, as for all protein fibres. If individual rings at the proximal ends of the threads are dissected off, opened flat, and examined between crossed nicols, it is possible to determine the arrangement of the fibres in the ring (fig. 5). Those which run down the stem

from the root-laminae are seen to end at right angles against those which run round the ring and are secreted by the byssus-gland lips. At the point of origin of the thread, the root fibres and the ring fibres converge and assume a course parallel to each other; together they run down into the thread, so that in the opened and flattened ring the fibres have a fan-like distribution, with the thread forming the handle of the fan.

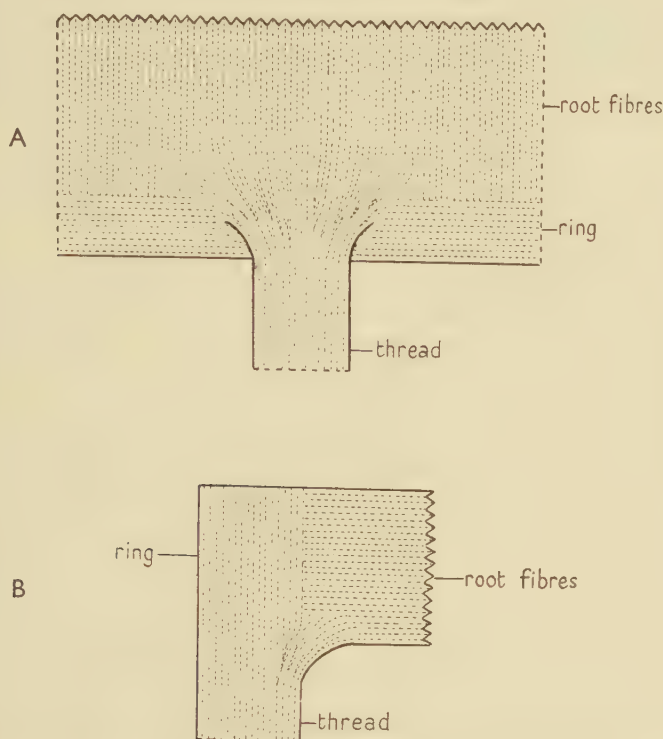


FIG. 5. Diagram to show the arrangement of the fibres in the byssus-thread and attachment-ring. A, attachment-ring cut through and opened out flat. B, side view of ring.

X-ray diffraction photographs of the byssus-threads of lamellibranchs have been made by Trogus and Hess (1933), Centrola (1936), and Champetier and Fauré-Fremiet (1938). The last showed that washed threads of *Mytilus* and *Pinna* give a photograph of the collagen-type, the reflections in which become sharper when the threads are stretched. X-ray photographs of both *Mytilus* and *Pinna* were taken during the present investigation and the findings of Champetier and Fauré-Fremiet were confirmed, the 2.9 Å meridional and the 11 Å and 4.5 Å equatorial spacings being clearly defined.

Chemical properties

The first published work on the chemical nature of the organic skeletal material in lamellibranchs was carried out by Fremy (1855), who examined the

residue after decalcification of the shell in various species. From estimates of the carbon, nitrogen, oxygen, and sulphur content of this material, he concluded that it was a well-defined organic substance, in many respects resembling collagen, but not yielding a soluble gelatin on heating in boiling water. He distinguished it from chitin on the basis of its nitrogen content. Because of its resistance to chemical reagents, he considered it to be closely related to the skeletal material of gorgonian corals, and he gave to it the name 'conchiolin'. Schlossberger (1856) and Krukenberg (1881–1882) made elementary analyses of *Mytilus* byssus material and compared it with conchiolin; they concluded that the two were identical. Abderhalden (1908) extended the analysis by acid hydrolysis and isolation of amino-acids. He found glycine, tyrosine, and proline in large quantities, as well as alanine and aspartic acid. Fauré-Fremiet and Boudony (1938) analysed the byssus material for nitrogen and sulphur. In view of the observed sulphur content of 0.76 per cent. they assumed that the material was a keratin.

All the standard protein colour-reactions are given by the byssus, while chitin tests are all negative, though the presence of traces of polysaccharide can be demonstrated by Molisch's test (see Cole, 1933). Neither pepsin nor trypsin digests the byssus after treatment for 24 hours.

No part of the byssus is soluble in water (cold or boiling), alcohol, ammonia, dilute acids or alkalis. It is slightly soluble in hot, concentrated acetic acid. In hot, concentrated sulphuric and hydrochloric acid the byssus first turns a wine-red colour and then dissolves—rapidly in sulphuric, more slowly in hydrochloric acid. In boiling, concentrated nitric acid the byssus dissolves to give a yellow solution. Complete solution also takes place in hot, concentrated potash.

Although the byssus is insoluble in boiling water, different parts undergo changes in dimensions, swelling in diameter and contracting in length, on boiling. Maximum shortening occurs between temperatures of 80° and 85° C., and is most marked in the byssus root, where the fibres shrink to about half their length. The contraction of the threads is much less than this. Various other oriented, fibrillar proteins, such as elastoidin (Picken, 1937), in which the protein chains are held together by electrovalent links and possibly van der Waals's attractions as well, show similar behaviour on heating in water. Thermal agitation breaks certain of the linkages and allows the protein chain-molecules to shorten; but breakage of all lateral linkages, leading to solution of the protein (as occurs with collagen) does not take place.

Swelling agents that break electrovalent links (such as aqueous lithium iodide) cause swelling of the root but have little or no effect on the rest of the byssus. Aqueous alkaline sodium sulphide, which dissolves keratin by breaking the electrovalent and disulphide bonds, will not dissolve the byssus; but aqueous sodium hypochlorite, which rapidly dissolves various quinone-tanned proteins (Slifer, 1945), dissolves all the byssus material.

The lead acetate test for sulphur gives only a faintly positive result, indicating that the sulphur content of the byssus is very low, and this observa-

tion, coupled with the failure of the byssus to dissolve in aqueous alkaline sodium sulphide, means that disulphide bonds (if present) must be few in number and can play but a small part in stabilizing the structure. The solubility of the byssus in aqueous sodium hypochlorite suggests that quinone-tanning may occur during its formation.

On soaking the byssus in a dilute solution of ferric chloride, the stem and threads turn greyish green and, after subsequent treatment with sodium carbonate, reddish mauve. This reaction is typical of orthodiphenols, which might act as precursors in quinone tanning. Accordingly, a histochemical study was made of the tissues of the foot responsible for the secretion of the byssus.

Anatomy of the foot

The first full description of the microscopic anatomy of the foot of *Mytilus* was given by Tullberg (1877). He described the external ciliated epithelium that covers the whole foot, and the posterior groove which widens into a sucker-like depression just before the distal end of the foot and runs up into the opening of the byssus gland at the junction of the foot and the visceral mass. The byssus-gland is a flask-shaped cavity, partitioned by numerous laminae, the plane of which is defined by the antero-posterior and dorso-ventral axes of the animal. Between these the sheets of material which form the root of the byssus are secreted. Tullberg also described the two main glands of the foot (fig. 6): first, the 'white' gland, which forms the bulk of the tissues of the foot, discharges into the trough of the groove along its whole length and extends up into the byssus-gland; second, the 'purple' gland. The latter is most voluminous round the depression at the distal end of the groove, where the attachment-disk is secreted, but it also extends on each side of the trough, along the length of the groove to the lips of the byssus-gland, but not into the byssus-gland itself. It discharges its secretion into the groove directly, through the epithelium; but ciliated ducts drain that portion of the gland which lies deeply embedded in the foot, anterior to the 'white' gland. These ducts discharge into the distal depression of the groove.

The groove varies in cross-section along its length, being much deeper and more slit-like just below the byssus-gland than it is nearer the distal end. On this may depend the difference in shape of cross-section between the proximal and distal parts of the threads. The attachment-disk is moulded by the sucker-like depression at the distal end of the foot and is formed largely from the secretion of the 'purple' gland. The root is formed exclusively of 'white' gland material.

The names of the glands of the foot are confusing. In fresh material it is possible to distinguish the 'purple' from the 'white' gland; but the 'purple' gland is in fact much whiter than the 'white' gland, which ranges in colour from cream to orange and is translucent in appearance. It is only in formaldehyde-fixed material that the 'purple' gland assumes a purple colour and thus becomes distinguishable by this colour from the 'white' gland.

It must be remembered that the foot has an extensive musculature, consisting of longitudinal, circular, and oblique muscles, concerned in the movements of the foot and probably also playing a part in squeezing the secretions of the 'white' and 'purple' glands into the posterior groove.

The most recent work on the histology of the glands is that of Turchini and Broussy (1934), but they, like Carrière and Cattie (1886), assumed that

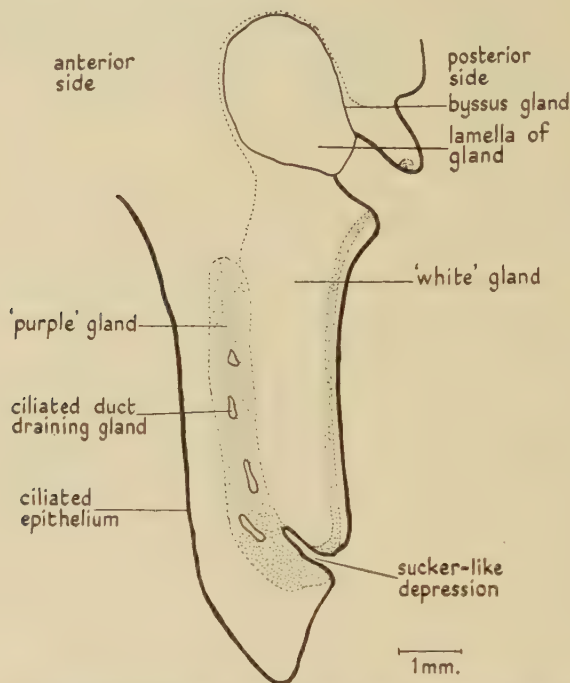


FIG. 6. Diagram of a longitudinal section through the foot of *Mytilus* slightly to one side of the posterior groove. The byssus has been removed.

there is only one gland secreting the byssus and that the 'purple' gland is a developmental stage of the 'white' gland.

Triple-staining methods, such as Azan or Masson, exhibit plainly the histologically different tissues of the foot. With Azan, the 'purple' gland stains a bright red, while the 'white' gland stains in shades of blue, intensely blue towards the distal end of the foot and a paler mauvish blue towards the byssus-gland. The contents of the 'purple' gland-cells are finely granular, but those of the 'white' gland contain large globules. The mode of secretion by the cells is of some interest. In both instances the gland-cells break down and liberate their contents into the intercellular spaces. The formative cells lie towards the periphery of the glands, while the centre of the glands and particularly those parts bordering the posterior groove are devoid of cells, but are packed with liberated granules which seem to be in process of being pressed towards the posterior groove. The epithelium bordering the trough

of the groove in the region of the 'white' gland is always very ill defined, and the 'white' gland secretion appears to be pushed out into the groove through the disorganized epithelium (fig. 7). The granules from the 'purple' gland, on the other hand, seem to be taken up by the epithelial cells and to pass out between the cilia, the epithelium always remaining well defined (figs. 7 and 9).

It was formerly believed that the material forming the byssus-thread came from the byssus-gland only, and that the posterior groove served merely as a mould. Sections of the foot at all levels show 'white' gland material being secreted into the groove in considerable quantities, and the total histological picture leaves no doubt that the thread is formed by secretion along the whole length of the groove. It seems that the 'white' gland material, after fusing to form a solid mass of material in the groove, is coated on the outside with a layer of material from the 'purple' gland, which fuses to form a continuous layer, and that the outer lips of the groove then mould these secretions into a rounded thread.

Pyefinch (1945) was the first to report that the 'purple' gland of *Mytilus* gave a positive argentaffin reaction (Lison, 1936), indicating the possible presence of polyphenols in the gland. This observation has been repeated, and a very clear picture has been obtained of the black argentaffin granules, both in the 'purple' gland and on their way through the groove-epithelium to form a border to the mass of 'white' gland material lying in the groove. In thick sections of fresh material treated with aqueous ferric chloride, the 'purple' gland is stained a greyish green which turns to purplish red on the addition of sodium carbonate. The diazo-reaction for phenols is positive: *p*-nitromethoxybenzodiazotate stains the 'purple' gland dark brown (see Brown, 1950). This staining can be inhibited by previous treatment with iodine. There is, therefore, considerable evidence for the presence of a phenol, almost certainly an orthodiphenol, in the 'purple' gland.

Since the 'purple' gland is relatively large, and *Mytilus* itself is readily obtainable in large numbers, an attempt was made to extract and identify the orthodiphenol present in the gland. It is not very difficult to dissect out the gland comparatively free from other material, and dissected glands were ground up in a variety of solvents. The filtered extracts, or the deposits left after evaporation of these, were then tested for the presence of polyphenols. The test for phenols used was the production of a red colour on treatment of the sample with nitrous acid followed by alkali (Castiglioni, 1932). Six to eight glands were used in each experiment, and where necessary the colours produced were examined in a comparator.

No polyphenol could be extracted from the gland with distilled water, alcohol, or ether, but extracts made with dilute sodium hydroxide or dilute hydrochloric acid contained polyphenols, the reaction being strongest with the acid extract, and enhanced by boiling.

In an acid extract neutralized with sodium hydroxide or ammonia, a purple, jelly-like precipitate appears at approximately pH 7.5. The precipitate gives positive phenol tests, and the supernatant liquid is free from phenols. On

neutralizing an alkaline extract, a precipitate giving positive phenol tests is also formed at pH 7.5, but in this case the precipitate is white. Neither heating an acid extract, nor adding alcohol, led to precipitation.

There is some evidence that tryptic digestion liberates the polyphenol from the 'purple' gland. After treating glands with trypsin for twenty-four hours, polyphenols could be detected in the fluid but not in the buffer controls.

Though similar quantities of wet gland, and similar volumes of extracting acid and neutralizing alkali, were used, the intensity of the colour of the precipitate was very variable in different experiments. The colour itself was not altered by treatment of the precipitate with reducing or oxidizing agents. If precipitation occurred in the presence of sodium sulphite (a reducing agent), there was no decrease in the intensity of the colour of the precipitate as compared with controls; but sodium nitrite and a trace of potassium cyanide both tended to inhibit the development of the colour, suggesting that the coloured precipitate is an oxidation product. It was not possible to convert an approximately white precipitate, obtained in this way, to a coloured precipitate by treatment with oxidizing agents. An increasingly dark precipitate was obtained by delaying the time of precipitation after extraction.

On evaporating an acid extract to dryness on a water bath, a brownish purple film of material was obtained. This was insoluble in hydrochloric acid, even on boiling.

Various protein colour-reactions, such as the ninhydrin, Millon, and biuret reactions, could be obtained from the precipitate, and these results, together with the solubility of the polyphenol in dilute acids and alkalis, and its insolubility at the neutral point, suggest that it is a phenolic amino-acid or protein, the isoelectric point of which lies at pH 7.5. It can be distinguished from tyrosine in that the precipitate gives a negative Mörners reaction (see Cole, 1933), and from dihydroxyphenylalanine (the only diphenolic amino-acid that has been described) in that unlike the latter it is insoluble in water. How far the extractable polyphenol is identical with the orthodiphenol as it exists in the 'purple' gland is unknown.

While the orthodiphenol itself might act as a tanning agent by forming electrovalent links with neighbouring protein molecules, the quinone obtained by oxidation of the orthodiphenol might be expected to give much more powerful tanning by the establishment of covalent links. Sections of the foot and portions of the byssus were therefore incubated with *l*-tyrosine in pH

FIG. 7 (plate). A, transverse section of the foot of *Mytilus* in the region of the groove, to show the secretion of the 'white' gland material (darkly stained) into the groove. Fixed Susa, stained Azan.

B, transverse section of the foot of *Mytilus* to show secretion of 'purple' gland material (shown black) into the groove. Fixed Susa, stained Azan.

C, transverse section of the foot of *Mytilus* to show the moulding of the secreted material by the outer portion of the groove (lower edge of photograph) to form the (dark) cord of the byssus thread. Central black cross-shaped secretion is thread in initial stages of formation, surrounded by 'white' gland tissue, with more darkly staining 'purple' gland below the cross. Fixed Susa, stained Azan.

to buffer, in order to test for the presence of a polyphenol oxidase, capable of oxidizing the orthodiphenol to a quinone. Sections of the foot itself did not blacken, but the medium in which the byssus samples were incubated rapidly went pink and very soon became black. These changes were inhibited by cyanide or by previously boiling the byssus in water. There was evidence, therefore, for the presence of a thermolabile polyphenol oxidase which might serve to oxidize the orthodiphenol to a quinone.

The similarity in colour of the precipitate from the acid extracts and of the older byssus-threads is very striking and suggests that the reason for the purple colour is the same or similar in both.

The 'white' gland gave all the protein colour reactions but did not give any of the polyphenol reactions.

This study of the byssus and the histochemistry of its formation suggests that the 'white' gland secretes globules of protein which, extruded between the laminae of the byssus gland, coalesce to form the fibrillar sheets of material which constitute the byssus root. Within individual laminae the protein chains are oriented parallel to the long axis of the byssus and they are bonded by electrovalent links and possibly by van der Waals's attractions, as is shown by the reactions of the material to swelling agents and to boiling. Those parts of the root-laminae which, on passing out of the byssus gland, are compressed by the lips of the gland and fuse with the secretions of the 'white' and 'purple' glands as they pass through the epithelium of the lips, form the hardened rings round the stem of the byssus. The threads themselves, moulded and tanned in the posterior groove, are attached to the rings from time to time; at which times the secretions of the byssus-gland, the gland-lips, and the posterior groove become confluent. Tanning may take place not only between the protein from the 'white' gland and the quinone derived from the 'purple' gland, but presumably also between the amino-portion and the quinone nucleus of adjacent molecules from the 'purple' gland.

The presence of an aromatic substance and a polyphenol oxidase system suggests that the mechanism of tanning is like that in the egg-case of the cockroach (Pryor, 1940); but the precursor of the tanning-agent in the cockroach is an alcohol-soluble polyphenol, protocatachuic acid (Pryor, Russell, and Todd, 1946). The aromatic substance in *Mytilus* is not alcohol-soluble, and its properties suggest that it is an amino-acid or protein. Until the polyphenol from *Mytilus* has been identified, it is not possible to state exactly how tanning takes place. It would be of interest to determine whether the oxidation to the quinone in *Mytilus* can occur without splitting the orthodiphenol into phenolic and amino-acid or protein fractions, as the evidence suggests. If the orthodiphenol is split off and oxidized separately, the mechanism of tanning would not be significantly different from that in insects; if this splitting off does not occur, however, the mechanism may be regarded as a more primitive method of tanning and may suggest a way in which the tanning mechanism has evolved. In the light of the present study, the material of the byssus is to be regarded as a scleroprotein in Pryor's sense (1940).

The byssus is well adapted for its purpose. *Mytilus*, living in the intertidal zone, is prevented from being thrown about as a result of wave- and tidal-action by the byssus which attaches it to the substratum. The strength and extensibility of the byssus-threads and their distribution in space, like guys about a flag-pole, allow the mussel to withstand both the pounding of the waves and the drag of the advancing and receding tides. It may remain fixed in one place for many months, and the high chemical resistance of the byssus will preserve it from decay. Should the mud which often collects on mussel-beds become deficient in oxygen, quinone-bonding would be an advantage over —S—S— bonding, since under these conditions the latter, but not the former, might become reduced; this would lead to a weakening of the thread. When *Mytilus* moves, it is able to pull itself free by strong contractions of the byssus-muscle, either breaking the byssus-threads or pulling itself away from the byssus at the root.

The periostracum, hinge, and organic ground-substance of the shell of Mytilus edulis

As we have seen already, the work of Fremy and Krukenberg suggested that there is a considerable similarity between the organic substance of the shell and the byssus of *Mytilus*.

Tullberg (1877) and Field (1922) have described the anatomy of the shell and its associated tissues, and it will suffice to recall that the shell is covered by a brown membrane, the periostracum, which in the dorsal mid-line expands to form a hinge between the two valves of the shell. The inorganic shell itself is permeated by organic material.

The periostracum

Material for investigating the microscopic structure of the periostracum was prepared for sectioning by fixing whole, small animals in Bouin's fluid or in 'Susa', decalcifying the shell in 1 per cent. nitric acid in 70 per cent. alcohol, and, after decalcification, clearing and embedding in paraffin wax under reduced pressure, to remove carbon dioxide bubbles formed during decalcification.

The mantle-edge ends in three lobes, and the periostracum originates between the outer and middle lobes (fig. 8). The periostracum is attached to the outer face of the middle lobe, but it is free from the inner face of the outer lobe. Field describes the periostracum as being secreted by an epithelial layer of the middle lobe, but no such epithelium can be seen in the region where the periostracum is attached to the middle lobe. Beyond the area of attachment of the periostracum, however, the middle lobe is covered by a columnar epithelium, and in the living animal this is pressed against the periostracum, though in sections it has retreated from the periostracum as a result of the contraction of muscles in the lobe. No definite gland-cells could be seen in this epithelium, however, and no evidence could be obtained that this, in fact, plays any part in the secretion of the periostracum. The

entire surface of the inner face of the outer lobe, on the other hand, is covered by a columnar epithelium with conspicuous nuclei, in which gland cells with granular contents abound. The granules stain red and blue with Azan and are reminiscent of the granules of the 'purple' and 'white' glands respectively, concerned in the formation of the byssus. Red and blue staining granules may also be seen lying in the space between the outer lobe and the periostracum. The histological evidence suggests, therefore, that the periostracum

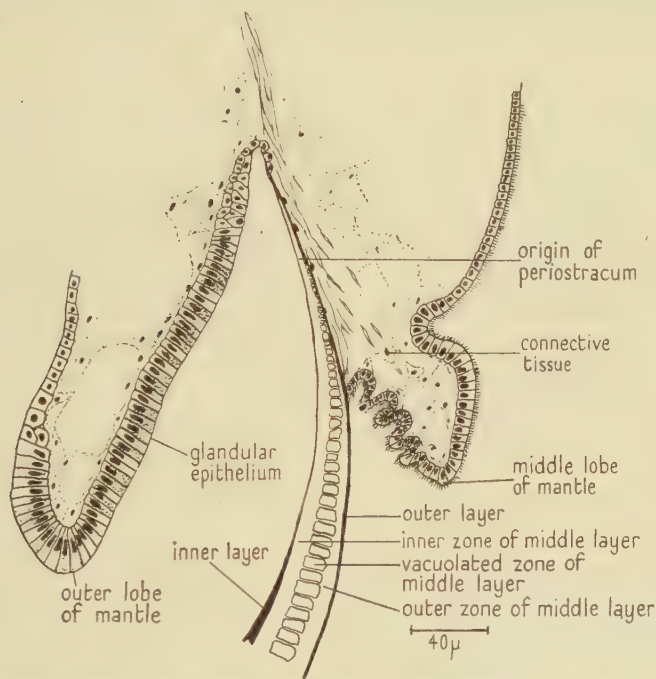


FIG. 8. Transverse section of the mantle-edge, showing the periostracum. Fixed Bouin, stained Masson's triple stain.

is secreted by the *inner face of the outer lobe* of the mantle edge, not, as Field describes, by the *middle lobe* of the mantle edge.

The periostracum itself is a complicated structure. Originating as a single homogeneous layer, it soon becomes differentiated into two and then three layers. The middle layer, that is, the first to be secreted, becomes differentiated into compact inner and outer zones separated by a vacuolated zone (fig. 8). In colour and appearance the middle layer resembles the freshly formed byssus threads, while the inner and outer layers are opaque and dark brown in colour.

In surface view, the middle layer is faintly positively birefringent with respect to the edge. The birefringence appears to be general and is not confined to the system of fibrils which can be seen running in the middle layer at right angles to the edge of the periostracum. In transverse sections

the middle layer is strongly birefringent, but the outer and inner layers are isotropic.

Argentaffin- and diazo-staining are both positive; the whole periostracum is stained an intense black by the argentaffin method. Black-staining granules also occur in the epithelium of the outer lobe of the mantle. The periostracum is composed, therefore, of a quinone-tanned protein (that is, a sclerotin), probably similar to that of the byssus, though the inner and outer layers may differ from the byssus, either physically or chemically. The periostracum never assumes the purple colour so often found in the byssus threads.

The hinge

A full description of the hinge of *Mytilus* has recently been given by Trueman (1950), so that only observations on the chemical nature of the hinge will be given here. The hinge is divided into two main layers, an inner and outer layer. Polyphenols were demonstrated in both layers of the hinge by the nitrous acid/alkali technique, indicating that it also is a quinone-tanned protein. Field (1922) commented on the calcium carbonate which occurs in the inner layer of the hinge. The outer layer was found to contain deposits of fat colourable by sudan black. As described by Trueman, the outer layer is subjected to a tensile strain transverse to the longitudinal plane of the shell, and this strain is increased when the valve is closed. The function of the fat is not known, but it may act as a lubricant of the protein molecules in the outer layer.

The ground substance of the shell

The organic ground-substance of the shell, which remains after decalcification, does not stain either with argentaffin- or diazo-stains. The material itself has a brown colour such as is often associated with the oxidation products of polyphenols. It is possible that any free polyphenols capable of giving the reaction are washed out during decalcification, leaving only the oxidation products combined in the tanned complex with the protein.

The supporting substance of the gills

The internal cavity of the gills of *Mytilus* is lined by a material which has been described as chitin by Peck (1877) and again by Kellog (1890). Field (1922), however, suggested that this material was similar to that of the byssus and periostracum.

The substance can be obtained free from adhering cells by soaking the gills in distilled water (Gray, 1920). The cells separate and wash away leaving the supporting substance in the form of combs of hollow tubes (fig. 9, A), two combs to each gill-plate, one supporting the descending limb, the other the ascending limb of the gill. The spines of the combs run along the free edge of the gill and the line of attachment of the gill to the body. Each spine gives off a series of hollow tubes, one running down each gill lamella. There is no direct connexion between the tube cavities of the descending and ascending

limbs; each tube ends blindly at the ventral edge of the gill and is connected to the opposite limb by strands of connective tissue only. It has not been possible to determine with certainty whether the cavity of the spine is continuous with the cavities of the lamella tubes.

The tubes show positive birefringence with respect to their length and the thickness difference is greatest in regions x and y (fig. 9, B), where the material is thickest.

All chitin tests on the material were negative. On treatment with ninhydrin, however, the material becomes blue in colour, indicating that it contains

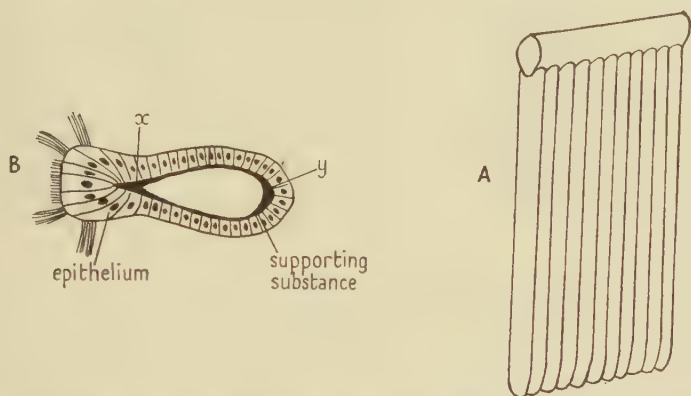


FIG. 9. A, diagram of the skeletal support to the descending limb of the gill of *Mytilus*. B, transverse section of a gill-lamina of *Mytilus* to show position of supporting material.

a protein. The tubes contract when boiled in distilled water, but do not dissolve; cold, dilute aqueous sodium hydroxide rapidly dissolves them, however. Swelling agents, such as aqueous lithium iodide, cause the tubes to contract spirally, but lateral swelling was not detectable. Tests for polyphenols all proved negative. In sections stained with Azan or Masson's triple stain, the material behaves like the axial portion of the byssus-threads and like the root of the byssus. The evidence so far available suggests that it is not a chitin but a fibrillar protein, the chains of which are held together by weak linkages (partly electrovalent), some of which are broken by thermal agitation in the presence of boiling water and all by dilute alkalis.

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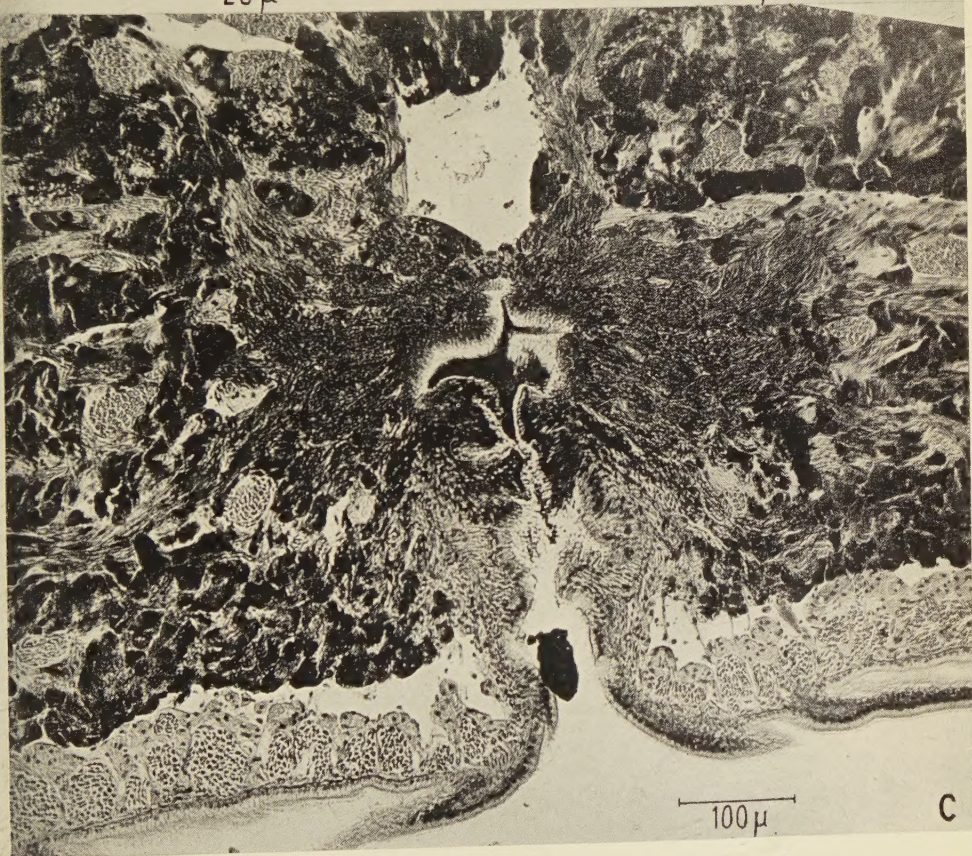


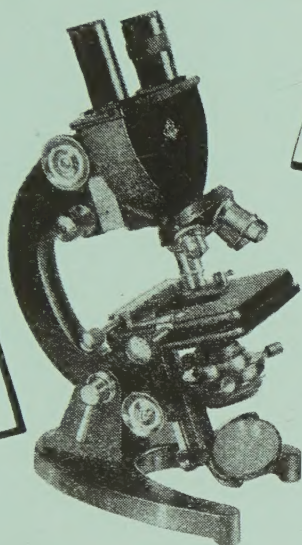
FIG. 7
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